08-12-08 1625

427.098

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:	
Lydie Poitout, et al.	:	Group: 1625
Serial No.: 10/550,122	:	
Filed: September 19, 2005	:	Examiner: Rahmani, Niloofar
For: IMIDAZOPYRIDINE... AGONISTS	:	

Hedman and Costigan
1185 Avenue of the Americas
New York, NY 10036
August 11, 2008

LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Supplemental to the letter of July 23, 2008, Applicants are submitting herewith additional documents cited in the declaration of Dr. Thurieau which were not supplied earlier since they were not available.

Respectfully submitted,

Charles A. Muserlian #19,683
Attorney for Applicants
Tel. 212 302 8989

CAM:mlp
Enclosures

"EXPRESS MAIL" Mailing Label Number : EH 288405578US

Date of Deposit: August 11, 2008

I hereby certify that this correspondence is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" Service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Marie-Louise Pinset

Hypothalamic peptides as drug targets for obesity

Waljit S Dhillo and Stephen R Bloom*

The importance of the melanocortin system in obesity has been confirmed by the recent discovery of mutations in the melanocortin MC_4 receptor in morbidly obese patients and the finding that intranasal administration of a fragment of melanocortin decreases body fat in humans. Transgenic mice overexpressing melanin-concentrating hormone (MCH) are obese and a second MCH receptor has been identified. In addition, ghrelin, endocannabinoids and glucagon-like peptide 2 have been identified as potentially important central regulators of food intake.

Addresses
6th Floor Commonwealth Building, Imperial College at Hammersmith Hospital, Du Cane Road, London W12 ONN, UK
*e-mail: s.bloom@ic.ac.uk

Current Opinion in Pharmacology 2001, 1:651–655

1471-4892/01/$ – see front matter

Abbreviations

Agrp	agouti-related protein
α-MSH	α melanocyte-stimulating hormone
CART	cocaine and amphetamine regulated transcript
GLP	glucagon-like peptide
ICV	intracerebroventricular
MCH	melanin-concentrating hormone
NPY	neuropeptide Y
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus

Introduction

Obesity is a major public health problem, being associated with the development of coronary heart disease and type 2 diabetes mellitus. The rate of increase of obesity is accelerating — obesity in England and Wales has increased from 6% of men and 8% of women in 1980 to 17% of men and 20% of women in 1997 [1]. The hypothalamus is the centre for the integration and control of feeding and satiety and the hypothalamic mechanisms involved in the control of feeding are now being unravelled. A number of novel hypothalamic neuropeptides have been identified that affect food intake and energy expenditure. These have become potential targets for drug development for the treatment of obesity. In this article, we review the recent advances in our understanding of the hypothalamic regulation of food intake and energy expenditure.

Hypothalamic circuits controlling food intake

The hypothalamic circuits that control food intake and energy expenditure are complex and involve a number of well-known peptides as well as recently identified hypothalamic peptides. Table 1 summarises hypothalamic orexigenic (increase food intake) and anorectic (decrease food intake) neuropeptides. Many of these neuropeptides affect energy expenditure as well as food intake, providing potential opportunities for the development of anti-obesity drugs.

Table 1

Orexigenic (increase food intake) and anorectic (decrease food intake) hypothalamic neuropeptides implicated in the control of food intake and energy homeostasis.

Orexigenic neuropeptides	Anorectic neuropeptides
Agrp	α-MSH
NPY	CART
Ghrelin	GLP-1, GLP-2, oxyntomodulin
MCH	
Endocannabinoids	

The melanocortin system

The melanocortin system is unique in that it consists of agonists, the melanocortins, and an endogenous antagonist, agouti-related protein (Agrp). There are five melanocortin receptors, of which only MC_3 and MC_4 are present in the brain. Melanocortins are produced by the proteolytic cleavage of the precursor molecule pro-opiomelanocortin (POMC) and exert their effects by agonism of the melanocortin receptors. Agrp is an endogenous antagonist of melanocortin MC_3 and MC_4 receptors. Evidence for the importance of the melanocortin system in the regulation of energy homeostasis is derived from a series of genetically modified mouse models of obesity. First, mice lacking MC_4 are hyperphagic and very obese [2]. Second, mice lacking POMC [3], and therefore having no α melanocyte-stimulating hormone (α-MSH), or mice overexpressing Agrp [4] are also obese. Overall this indicates that tonic signalling by melanocortin MC_4 receptors acts to limit food intake. Additional support comes from *in vivo* studies in which intracerebroventricular (ICV) administration of α-MSH reduces food intake, whereas Agrp increases food intake [5]. The hypothalamic nuclei also vary in their sensitivity to Agrp and α-MSH with regard to their effect on feeding. The paraventricular nucleus (PVN), dorsomedial hypothalamus and the medial pre-optic areas have the greatest response to the feeding effects of Agrp and α-MSH ([6]; see also Update).

The importance of this system in man is confirmed by the identification of inactivating mutations in the human POMC [7] and MC_4 receptor [8] genes in extremely obese children. Recently, a high frequency (4%) of heterozygous MC_4 receptor mutations was reported in a large population of morbidly obese patients, with no such mutations found in controls. These patients displayed a common, nonsyndromic form of obesity [9•].

Further evidence that activation of the MC_4 receptor is a physiological inhibitor of feeding is suggested by a report that altered energy balance causes selective changes in MC_4 receptors in specific hypothalamic regions known to be

important in the control of food intake. After 10 days of food restriction, the density of MC_4 receptors was significantly increased by 20–65% in the ventromedial and arcuate nuclei and dorsomedial hypothalamus, with no changes elsewhere. In contrast, rats with diet-induced obesity showed significantly decreased density of MC_4 receptors, especially in the ventromedial and arcuate nuclei [10]. The melanocortin MC_4 receptor is also important in the control of energy expenditure. Intraperitoneal administration of the non-selective melanocortin receptor agonist MTII increases metabolic rate in wild-type mice but not in MC_4 receptor knockout mice [11]. The central melanocortin system has also been shown to affect the hypothalamic–pituitary–thyroid (HPT) axis. The hypothalamic melanocortins can stimulate the thyroid axis and Agrp can inhibit it, suggesting that the hypothalamic melanocortin system could influence energy expenditure through the HPT axis [12]. Further evidence that Agrp has a role in energy expenditure comes from a recent report that chronic Agrp treatment decreases interscapular brown adipose tissue uncoupling protein 1, suppresses plasma thyroid stimulating hormone and increases epididymal fat pad weight, independently of its orexigenic effects [13].

On the basis of this knowledge of the melanocortin system, melanocortin MC_4 receptor agonists are being developed as potential treatments for obesity. Recently, it has been shown that a fragment of melanocortin ($MSH/ACTH_{4-10}$), which represents the core sequence of all melanocortins, can modestly decrease body fat in humans. Normal weight subjects given $MSH/ACTH_{4-10}$ intranasally twice daily for 6 weeks had reduced body fat (1.68 kg) and body weight (0.79 kg) compared with placebo-treated subjects [14].

Neuropeptide Y

Neuropeptide Y (NPY) is a powerful orexigenic neuropeptide. ICV administration of NPY to rats stimulates food intake and repeated ICV administration of NPY readily leads to obesity [15]. PVN secretion of NPY increases in association with increased appetite [16]. There are five known NPY receptors, two of which (Y_1 and Y_5 receptors) have been postulated to mediate the orexigenic effects of NPY. A number of NPY Y_1 and Y_5 receptor antagonists have therefore been evaluated as potential therapeutic agents for obesity [17]. The Y_5 receptor has also been implicated in energy expenditure regulation. ICV administration of NPY or Y_5 receptor selective agonists decreased energy expenditure by reducing brown adipose tissue and temperature, and reducing oxygen consumption [18]. NPY can also affect energy expenditure more indirectly by central inhibition of the thyroid axis [19].

The complexity of hypothalamic systems is emphasised by the finding that mice lacking NPY, Y_1 or Y_5 have intact feeding responses. This suggests that other systems can replace NPY in its absence in some circumstances; however, Y_2 receptor null mice develop increased body weight, food intake and fat deposition while maintaining a normal response to NPY-induced food intake [20]. This suggests that the Y_2 receptor has an inhibitory role in the central regulation of body weight and control of food intake.

Ghrelin

Growth hormone secretagogues (GHSs) are small synthetic molecules that stimulate the release of growth hormone from the pituitary. They act through the GHS receptor (GHS-R), a G-protein-coupled receptor for which the ligand was unknown until ghrelin (a 28 amino acid peptide) was identified as its endogenous ligand [21]. Ghrelin-immunoreactive cells are located in the arcuate nucleus of the hypothalamus and the stomach. As expected, ghrelin stimulates the release of growth hormone both *in vivo* and *in vitro*.

ICV administration of ghrelin causes a dose-dependent increase in food intake and body weight. Peripheral daily administration of ghrelin causes weight gain by reducing fat utilisation in mice and rats. Rat serum ghrelin concentrations are increased by fasting and reduced by re-feeding or oral glucose administration, but not by water ingestion [22,23•].

The orexigenic effects of ghrelin may in part be mediated via other neuropeptides, for example Agrp and NPY. After the ICV administration of ghrelin, Fos protein, a marker of neuronal activation, was found in regions containing NPY and Agrp neurons. Co-administration of antibodies and antagonists of NPY and Agrp abolished ghrelin-induced feeding [24]. The orexigenic effects of ghrelin are also abolished dose dependently by ICV co-administration of an NPY Y_1 receptor antagonist [25]. Central administration of ghrelin increased both Agrp mRNA levels [26] and NPY mRNA levels [25] in the hypothalamus. As ghrelin increases food intake in rats that are genetically deficient in growth hormone [24,25], it is unlikely that the feeding effects of ghrelin are mediated by an increase in growth hormone. Circulating ghrelin levels have been shown to be decreased in human obesity. Plasma ghrelin concentration was lower in obese Caucasians and Pima Indians (a population with a very high prevalence of obesity) compared with a lean control population [27•].

Melanin-concentrating hormone

Melanin-concentrating hormone (MCH) is an orexigenic neuropeptide found in the lateral hypothalamus. Fasting increases the expression of MCH mRNA in normal and obese animals. The injection of MCH into the lateral ventricles of rats has been shown to increase food intake acutely [28], but tolerance was seen with chronic administration [29]. An important role for MCH in the control of food intake is demonstrated by MCH knockout mice and MCH overexpressing mice. MCH-deficient mice have reduced body weight and leanness due to hypophagia (reduced feeding) and an inappropriately increased metabolic rate [30], suggesting a role for MCH in the central regulation of food intake and energy expenditure. Transgenic mice that overexpress MCH in the lateral

hypothalamus, at approximately twofold higher levels than in normal mice, are obese and insulin resistant [31]. Homozygous transgenic animals fed a high-fat diet ate 10% more and were 12% heavier at 13 weeks of age than wild-type animals. The role of MCH in the control of energy expenditure is emphasised by a recent report that MCH suppresses release of thyroid-stimulating hormone [32]. Two G-protein-coupled receptors have recently been identified as the MCH receptors [33,34•], providing new potential drug targets.

Endocannabinoids

Anandamide and 2-arachidonoyl glycerol are endogenous cannabinoids (endocannabinoids) that are present in the hypothalamus. The cannabinoid CB_1 receptor is a major target for the action of cannabinoids in the central nervous system. Subcutaneous injections of anandamide induces significant overeating in satiated rats and this effect is dose-dependently attenuated by pretreatment with the selective CB_1 receptor antagonist SR141716 [35]. Chronic administration of SR141716 to adult, non-obese Wistar rats dose-dependently reduces both food intake and body weight; however, tolerance to the anorectic effect develops within 5 days [36]. CB_1 receptor knockout mice ($CB_1^{-/-}$) eat less than their wild-type littermates following 18 hours of fasting. Wild-type mice fasted for 18 hours and treated with the selective CB_1 receptor antagonist SR141716 have significantly reduced food intake, consuming the same amount as vehicle-treated $CB_1^{-/-}$ mice. These results indicate that endogenous cannabinoids, acting at CB_1 receptors, may be involved in maintaining food intake in mice made hyperphagic by brief food deprivation. In non-fasted animals, food intake is similar in $CB_1^{+/+}$ and in $CB_1^{-/-}$ mice, suggesting that in the absence of food deprivation, other orexigenic signals compensate for the loss of endocannabinoid signalling [37••].

Cocaine and amphetamine regulated transcript

Hypothalamic cocaine and amphetamine regulated transcript (CART) is thought to be an important anorectic peptide [38,39]. When injected ICV into rats, CART inhibits both normal and starvation-induced feeding. Endogenous CART may exert an inhibitory tone on feeding because injection of an antibody to CART peptide increases feeding in normal rats. Food-deprived animals show a decrease in expression of CART mRNA in the arcuate nucleus; however, the ICV administration of CART has been reported to be associated with some abnormalities in behaviour, particularly movement-associated tremors [38,40]. This suggests that the inhibition of feeding seen following CART administration is an adverse effect rather than a true anorectic effect. In addition, it has recently been demonstrated that injection of CART into discrete hypothalamic nuclei of 24-hour fasted rats results in a significant increase in feeding [41], without any of these behavioural abnormalities. Thus, the true role of CART in the regulation of food intake is unclear. Overall, these data might suggest that there are two types of CART appetite circuits within the hypothalamus, one orexigenic and the other anorectic.

Gluagon-like peptides

Glucagon-like peptides (GLPs) are produced by the proteolytic cleavage of proglucagon in intestinal L cells and in the central nervous system, and include GLP-1, GLP-2 and oxyntomodulin. The ICV administration of GLP-1 powerfully inhibits feeding in fasted rats [42]. The ICV injection of the specific GLP-1 receptor antagonist exendin (9–39), blocks the inhibitory effect of GLP-1 on food intake. Exendin (9–39) alone has no influence on fast-induced feeding but more than doubles food intake in satiated rats [42]; however, GLP-1 receptor knockout mice exhibit glucose intolerance with normal feeding behaviour [43], suggesting that GLP-1 may be more important in the regulation of glycaemia than food intake. The infusion of GLP-1 in healthy human volunteers results in a decrease in fasting glucose, which can be blocked by co-administration of exendin [44]. In humans, the infusion of GLP-1 also causes a reduction in spontaneous food intake [45].

GLP-2 has also been reported to reduce food intake in rats. The ICV injection of GLP-2 to rats after an overnight fast results in a 35% decrease in 2-hour food intake, and the ICV injection of GLP-2 to rats before the onset of dark (rats eat most in the dark) reduces food intake at 2 hours [46••].

Recently, oxyntomodulin has been reported to inhibit food intake. The administration of oxyntomodulin ICV or into the PVN after a 24-hour fast or at the onset of the dark phase causes a reduction in food intake. This anorectic effect of oxyntomodulin on feeding is of a similar magnitude and time course as an equimolar dose of GLP-1 [47].

Conclusions

The accelerating epidemic of obesity is threatening the health of the Western world; however, our ability to unravel the mechanisms in the brain that cause obesity will allow us to develop treatments to control it. Identification of genetic defects in human POMC and the melanocortin MC_4 receptor in obese individuals confirms the importance of the melanocortin system in the control of food intake and energy expenditure. Ghrelin, MCH and GLP-2 are exciting novel hypothalamic neuropeptides that are implicated in the development of obesity; however, while neurophysiologists forge ahead with the discovery of new neuropeptides that may play a role in the development of obesity, we rely on the genetic analysis of overweight populations and the development of selective drugs to determine which of these neuropeptides are important in the pathogenesis of obesity.

Update

Recent work has suggested that syndecan-3 physiologically modulates feeding behaviour, via the melanocortin system. Syndecan-3 is a member of a family of transmembrane heparan sulfate proteoglycans that may modulate

ligand–receptor interactions. Syndecan-3 is expressed in the hypothalamus and potentiates the inhibitory action of Agrp at the MC_4 receptor *in vitro*. Fasting increases hypothalamic syndecan-3 levels several-fold and Syndecan-3 null mice show markedly reduced reflex hyperphagia in response to fasting [48••].

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Prescott-Clarke P, Primatesta P: *Health Survey for England 1996.* London: HMSO; 1998.
2. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD *et al.*: **Targeted disruption of the melanocortin-4 receptor results in obesity in mice.** *Cell* 1997, 88:131-141.
3. Yaswen L, Diehl N, Brennan MB, Hochgeschwender U: **Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin.** *Nat Med* 1999, 5:1066-1070.
4. Graham M, Shutter JR, Sarmiento U, Sarosi I, Stark KL: **Overexpression of Agrt leads to obesity in transgenic mice.** *Nat Genet* 1997, 17:273-274.
5. Rossi M, Kim MS, Morgan DG, Small CJ, Edwards CM, Sunter D, Abusnana S, Goldstone AP, Russell SH, Stanley SA *et al.*: **A C-terminal fragment of agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone *in vivo*.** *Endocrinology* 1998, 139:4428-4431.
6. Kim MS, Rossi M, Abusnana S, Sunter D, Morgan DG, Small CJ, Edwards CM, Heath MM, Stanley SA, Seal LJ *et al.*: **Hypothalamic localization of the feeding effect of agouti-related peptide and alpha-melanocyte-stimulating hormone.** *Diabetes* 2000, 49:177-182.
7. Krude H, Biebermann H, Luck W, Horn R, Brabant G, Gruters A: **Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans.** *Nat Genet* 1998, 19:155-157.
8. Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, O'Rahilly S: **A frameshift mutation in MC4R associated with dominantly inherited human obesity.** *Nat Genet* 1998, 20:111-112.
9. • Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B, Froguel P: **Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity.** *J Clin Invest* 2000, 106:253-262.

This article reports that a high frequency of rare heterozygous MC_4 receptor mutations occurs in extremely obese individuals.

10. Harrold JA, Widdowson PS, Williams G: **Altered energy balance causes selective changes in melanocortin-4 (MC4-R), but not melanocortin-3 (MC3-R), receptors in specific hypothalamic regions: further evidence that activation of MC4-R is a physiological inhibitor of feeding.** *Diabetes* 1999, 48:267-271.
11. Chen AS, Metzger JM, Trumbauer ME, Guan XM, Yu H, Frazier EG, Marsh DJ, Forrest MJ, Gopal-Truter S, Fisher J *et al.*: **Role of the melanocortin-4 receptor in metabolic rate and food intake in mice.** *Transgenic Res* 2000, 9:145-154.
12. Kim MS, Small CJ, Stanley SA, Morgan DG, Seal LJ, Kong WM, Edwards CM, Abusnana S, Sunter D, Ghatei MA, Bloom SR: **The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin.** *J Clin Invest* 2000, 105:1005-1011.
13. Small CJ, Kim MS, Stanley SA, Mitchell JR, Murphy K, Morgan DG, Ghatei MA, Bloom SR: **Effects of chronic central nervous system administration of agouti-related protein in pair-fed animals.** *Diabetes* 2001, 50:248-254.
14. Fehm HL, Smolnik R, Kern W, McGregor GP, Bickel U, Born J: **The melanocortin melanocyte-stimulating hormone/adrenocorticotropin(4–10) decreases body fat in humans.** *J Clin Endocrinol Metab* 2001, 86:1144-1148.
15. Zarjevski N, Cusin I, Vettor R, Rohner-Jeanrenaud F, Jeanrenaud B: **Chronic intracerebroventricular neuropeptide-Y administration to normal rats mimics hormonal and metabolic changes of obesity.** *Endocrinology* 1993, 133:1753-1758.
16. Kalra SP, Dube MG, Sahu A, Phelps CP, Kalra S: **Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food.** *Proc Natl Acad Sci USA* 1991, 88:10931-10935.
17. Duhault J, Boulanger M, Chamorro S, Boutin JA, Della ZO, Douillet E, Fauchere JL, Feletou M, Germain M, Husson B *et al.*: **Food intake regulation in rodents: Y5 or Y1 NPY receptors or both?** *Can J Physiol Pharmacol* 2000, 78:173-185.
18. Hwa JJ, Witten MB, Williams P, Ghibaudi L, Gao J, Salisbury BG, Mullins D, Hamud F, Strader CD, Parker EM: **Activation of the NPY Y5 receptor regulates both feeding and energy expenditure.** *Am J Physiol* 1999, 277:R1428-R1434.
19. Fekete C, Kelly J, Mihaly E, Sarkar S, Rand WM, Legradi G, Emerson CH, Lechan RM: **Neuropeptide Y has a central inhibitory action on the hypothalamic-pituitary-thyroid axis.** *Endocrinology* 2001, 142:2606-2613.
20. Naveilhan P, Hassani H, Canals JM, Ekstrand AJ, Larefalk A, Chhajlani V, Arenas E, Gedda K, Svensson L, Thoren P, Ernfors P: **Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y2 receptor.** *Nat Med* 1999, 5:1188-1193.
21. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: **Ghrelin is a growth-hormone-releasing acylated peptide from stomach.** *Nature* 1999, 402:656-660.
22. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR: **The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion.** *Endocrinology* 2000, 141:4325-4328.
23. • Tschop M, Smiley DL, Heiman ML: **Ghrelin induces adiposity in rodents.** *Nature* 2000, 407:908-913.

The authors demonstrate that central administration of ghrelin causes an increase in food intake and body weight.

24. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S: **A role for ghrelin in the central regulation of feeding.** *Nature* 2001, 409:194-198.
25. Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M *et al.*: **Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway.** *Diabetes* 2001, 50:227-232.
26. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I: **Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression.** *Endocrinology* 2000, 141:4797-4800.
27. • Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML: **Circulating ghrelin levels are decreased in human obesity.** *Diabetes* 2001, 50:707-709.

This is the first report that circulating ghrelin levels are decreased in human obesity.

28. Ou D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes WF, Przypek R, Kanarek R, Maratos-Flier E: **A role for melanin-concentrating hormone in the central regulation of feeding behaviour.** *Nature* 1996, 380:243-247.
29. Rossi M, Choi SJ, O'Shea D, Miyoshi T, Ghatei MA, Bloom SR: **Melanin-concentrating hormone acutely stimulates feeding, but chronic administration has no effect on body weight.** *Endocrinology* 1997, 138:351-355.
30. Shimada M, Tritos NA, Lowell BB, Flier JS, Maratos-Flier E: **Mice lacking melanin-concentrating hormone are hypophagic and lean.** *Nature* 1998, 396:670-674.
31. Ludwig DS, Tritos NA, Mastaitis JW, Kulkarni R, Kokkotou E, Elmquist J, Lowell B, Flier JS, Maratos-Flier E: **Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance.** *J Clin Invest* 2001, 107:379-386.
32. Kennedy AR, Todd JF, Stanley SA, Abbott CR, Small CJ, Ghatei MA, Bloom SR: **Melanin-concentrating hormone (MCH) suppresses thyroid stimulating hormone (TSH) release, *in vivo* and *in vitro*, via**

the hypothalamus and the pituitary. *Endocrinology* 2001, 142:3265-3268.

33. Chambers J, Ames RS, Bergsma D, Muir A, Fitzgerald LR, Hervieu G, Dytko GM, Foley JJ, Martin J, Liu WS *et al.*: Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. *Nature* 1999, 400:261-265.

34. • Mori M, Harada M, Terao Y, Sugo T, Watanabe T, Shimomura Y, Abe M, Shintani Y, Onda H, Nishimura O, Fujino M: Cloning of a novel G protein-coupled receptor, SLT, a subtype of the melanin-concentrating hormone receptor. *Biochem Biophys Res Commun* 2001, 283:1013-1018.

This is the first report of the identification of a second MCH receptor.

35. Williams CM, Kirkham TC: Anandamide induces overeating: mediation by central cannabinoid (CB1) receptors. *Psychopharmacology* 1999, 143:315-317.

36. Colombo G, Agabio R, Diaz G, Lobina C, Reali R, Gessa GL: Appetite suppression and weight loss after the cannabinoid antagonist SR 141716. *Life Sci* 1998, 63:L113-L117.

37. •• Di Marzo VV, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T, Kunos G: Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* 2001, 410:822-825.

This report demonstrates that endogenous cannabinoids may be involved in maintaining food intake in mice made hyperphagic by brief food deprivation.

38. Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N *et al.*: Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 1998, 393:72-76.

39. Lambert PD, Couceyro PR, McGirr KM, Dall Vechia SE, Smith Y, Kuhar MJ: CART peptides in the central control of feeding and interactions with neuropeptide Y. *Synapse* 1998, 29:293-298.

40. Vrang N, Tang-Christensen M, Larsen PJ, Kristensen P: Recombinant CART peptide induces c-Fos expression in central areas involved in control of feeding behaviour. *Brain Res* 1999, 818:499-509.

41. Abbott CR, Rossi M, Wren AM, Murphy KG, Kennedy AR, Stanley SA, Zollner AN, Morgan DG, Morgan I, Ghatei MA *et al.*: Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript (CART) following administration into discrete hypothalamic nuclei. *Endocrinology* 2001, 142:3457-3463.

42. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD *et al.*: A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature* 1996, 379:69-72.

43. Scrocchi LA, Brown TJ, MaClusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ: Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nat Med* 1996, 2:1254-1258.

44. Edwards CM, Todd JF, Mahmoudi M, Wang Z, Wang RM, Ghatei MA, Bloom SR: Glucagon-like peptide 1 has a physiological role in the control of postprandial glucose in humans: studies with the antagonist exendin 9–39. *Diabetes* 1999, 48:86-93.

45. Gutzwiller JP, Goke B, Drewe J, Hildebrand P, Ketterer S, Handschin D, Winterhalder R, Conen D, Beglinger C: Glucagon-like peptide-1: a potent regulator of food intake in humans. *Gut* 1999, 44:81-86.

46. •• Tang-Christensen M, Larsen PJ, Thulesen J, Romer J, Vrang N: The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake. *Nat Med* 2000, 6: 802-807.

This is the first report of the anorectic effects of GLP-2.

47. Dakin CL, Gunn I, Small CJ, Edwards CMB, Hay DL, Smith DM, Ghatei MA, Bloom SR: Oxyntomodulin inhibits food intake in the rat. *Endocrinology* 2001, in press.

48. •• Reizes O, Lincecum J, Wang Z, Goldberger O, Huang L, Kaksonen M, Ahima R, Hinkes MT, Barsch GS, Rauvala H, Bernfield M: Transgenic expression of Syndecan-1 uncovers a physiological control of feeding behaviour by Syndecan-3. *Cell* 2001, 106:105-116.

This report suggests that Syndecan-3 potentiates the inhibitory action of Agrp at the MC_4 receptor to physiologically modulate feeding behaviour.

Melanocortin-4 Receptor Antagonists for the Treatment of Depression and Anxiety Disorders

Shigeyuki Chaki* and Taketoshi Okubo

Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-cho, Kita-ku, Saitama, Saitama 331-9530, JAPAN

Abstract: Derived from proopiomelanocortin by proteolytic processing, melanocortins have been implicated in a wide range of physiological processes. Melanocortins exert their physiological effects by binding to specific receptors on the surface of cell membranes. To date, five subtypes of melanocortin receptors (MC1 – MC5) have been identified, all of which are G-protein coupled receptor whose activation leads to increase in intracellular cyclic 3',5'-adenosine monophosphate formation. Of these, the MC4 receptor is expressed predominantly throughout the central nervous system, particularly, in areas related to stress responses and emotional states. Expression of the MC4 receptor is regulated by stress exposure. Reports also indicate that stimulation of the MC4 receptor activates the hypothalamus-pituitary-adrenal axis, and that the MC4 receptor mediates stress-related behaviors and anxiety in rodents.

Recently developed selective MC4 receptor antagonists have demonstrated antidepressant and anxiolytic effects in several animal models of depression and anxiety. MC4 receptor antagonists are effective, particularly under conditions of high stress, which may be consistent with the etiology of depression and anxiety. This review describes the involvement of the MC4 receptor in stress response and discusses the potential value of MC4 receptor antagonists for the treatment of stress-related disorders such as depression and anxiety.

Keywords: MC4 receptor, melanocortin, antidepressant, anxiolytic, depression, anxiety, hypothalamus-pituitary-adrenal axis.

1. THE MELANOCORTIN SYSTEM

Melanocortins (Adrenocorticotropin (ACTH) and α-, β-, and γ-melanocyte-stimulating hormone (α-, β-, γ-MSH)) are derived from proopiomelanocortin (POMC) by enzymatic processing using prohormone convertases PC1 and PC2. The involvement of melanocortins in a wide range of physiological functions is well-documented, including feeding behavior [1], regulation of energy balance [2], thermoregulation [3], pain threshold [4], inflammation [5] and pigmentation [6]. The POMC gene is expressed primarily in the central nervous system and in the pituitary. In the brain, POMC cell bodies are found in the arcuate nucleus of the hypothalamus, and in the nucleus of the solitary tract in the brainstem [7]. Melanocortinergic immunoreactivity is detected throughout the brain including several hypothalamic nuclei such as the arcuate nucleus, paraventricular nucleus (PVN), dorsomedial hypothalamic nucleus, and lateral hypothalamic area [8]. In the PVN, α-MSH and ACTH immunoreactivity has been detected in the parvocellular and magnocellular subdivisions [9]. In addition, melanocortinergic immunoreactivity is observed in the limbic system, including the central nucleus of the amydgala and lateral septum [8]. Reports also suggest that α-MSH terminals in the central nucleus of amygdala are originated, at least in part, from the arcuate nucleus of the hypothalamus [10]. Thus, the brain melanocortin system is distributed in regions related to stress and emotional states. Moreover, it has been reported that melanocortinergic immunoreactivity is detected in the ventral tegmental area, dorsal raphe nucleus and locus coeruleus [8], suggesting interaction with monoaminergic neurons.

Five subtypes of melanocortin receptors have been reported (MC1 receptor – MC5 receptor), all Gs protein-coupled receptors. These receptors have different pharmacological and physiological roles. The MC1, MC3, MC4, and MC5 receptors are activated both by ACTH and MSH peptides, whereas the MC2 receptor is activated by ACTH but not by MSH peptides. α-MSH has higher affinity for MC1, MC4 and MC5 receptors than β- and γ-MSH, while the MC3 receptor has roughly the same affinity for these MSH peptides. Agouti, an endogenous melanocortin receptor antagonist, binds to MC1 and MC4 receptors but not to MC3 and MC5 receptors [11], while agouti-related protein, another endogenous melanocortin receptor antagonist, antagonizes MC3 and MC4 receptors [12]. The MC1 receptor is expressed on cutaneous melanocytes, macrophages and monocytes [13], and may be involved in skin and hair pigmentation and the anti-inflammatory action of melanocortins. The MC2 receptor is expressed in the cortex of the adrenal gland where it mediates the effects of ACTH on steroid secretion [14]. The MC3 receptor is expressed in the brain and placenta [15], while MC5 is distributed ubiquitously in peripheral tissue, with limited expression in the brain [16]. In addition to the MC4 receptor, the MC3 receptor is expressed in the brain predominantly in hypothalamic nuclei, including the ventromedial nucleus and arcuate nucleus, which are involved in feeding and energy expenditure [17]. In accord with this distribution, it has been suggested that the MC3 receptor is deeply involved in energy homeostasis [18].

2. MC4 RECEPTOR: MOLECULAR PROPERTIES AND DISTRIBUTION IN THE BRAIN

Human MC4 receptor cDNA was first cloned in 1993 [19]. The human MC4 receptor is a 332 amino acid protein encoded by a single exon of 999 nucleotides, and agonist stimulation leads to increase in intracellular cyclic 3',5'-adenosine monophosphate (cAMP) formation [19]. The human MC4 receptor is structurally most similar to the MC3 receptor, with which it exhibits 58% and 76% overall amino acid identity and similarity, respectively [19]. No significant species differences in molecular and pharmacological properties between human and rat MC4 receptors have been reported [20].

MC4 receptor mRNA is located mainly in the central nervous system, with multiple sites of expression including the cortex, thalamus, hypothalamus, brainstem and spinal cord [21,22], while the MC4 receptor has not been detected in peripheral tissues in studies covering 20 human organs [23]. In the brain, expression of MC4 receptor is found in the limbic system, i.e., in several nuclei of the amygdala including the central and basolateral nuclei, as well as in the lateral septal nucleus, hippocampus and enthorhinal cortex [22]. Thus, the MC4 receptor may be involved in regulation of emotional states. The MC4 receptor is found in both the parvocellular and magnocellular of the PVN of the hypo-thalamus, suggesting that it plays a role in the regulation of the activity of the hypothalamus-pituitary-adrenal (HPA) axis via arginine vasopressin

*Address correspondence to this author at the Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-cho, Kita-ku, Saitama, Saitama 331-9530, Japan; Tel: +81-48-669-3089; Fax: +81-48-652-7254; E-mail: s.chaki@po.rd.taisho.co.jp

1568-0266/07 $50.00+.00

(AVP) and corticotropin-releasing factor (CRF) neurons [22]. Notably, the MC3 receptor, another major melanocortin receptor subtype in the hypothalamic region, is not detected in the PVN of hypothalamus, suggesting that the MC3 receptor may not mediate regulation of the HPA axis. Overlaps between expression of the MC4 receptor and the monoaminergic systems have also been found. The MC4 receptor is expressed in the dorsal raphe nucleus (DRN) and locus coeruleus (LC), suggesting that the MC4 receptor may modulate the activity of serotonergic and noradrenergic systems [21,22]. This hypothesis is supported by our earlier findings that stimulation of MC4 receptor with MT II, an MC4 receptor agonist, alters the neuronal activity of both serotonin and noradrenaline neurons by single cell recording [24]. In addition, the MC4 receptor is densely expressed in the caudate putamen, core and shell of the nucleus accumbens as well as in the ventral tegmental area and substantia nigra [21]. The MC4 receptor is thus expressed in dopaminergic nuclei as well as in the main dopaminergic projection areas.

3. INVOLVEMENT OF THE MC4 RECEPTOR IN STRESS RESPONSES AND ANXIETY

3.1. Involvement in Stress-Related Behavior

It has been reported that α-MSH and ACTH induce excessive grooming behavior in rats [25,26], regarded as a rodent behavioral response to stress, while serum to ACTH reduces novelty-induced grooming [27]. Moreover, it has been reported that MT II, an MC4 receptor agonist, potently induces grooming in rats, while Nle-γ-MSH, a preferable MC3 agonist, does not [25]. Additionally, SHU9119, an MC4/MC3 receptor antagonist, has been reported to attenuate both MT II- and novelty-induced excessive grooming [25]. On this basis, it has been suggested that grooming behavior induced by melanocortins as well as by novel environments (stress) is mediated through activation of the MC4 receptor, but not through the MC3 receptor.

Exposure to stress induces various behavioral abnormalities, including reduced feeding behavior. It has been reported that the selective MC4 receptor antagonist HS014 blocks immobility stress-induced anorexia in rats [28]. Likewise, we have previously reported that MCL0020, a selective peptidemimetic MC4 receptor antagonist attenuates restraint stress-induced reduction of food intake in fasted rats at doses at which it fails to affect food intake induced by fasting alone [29]. It appears that melanocortins may induce stress-related behaviors in rodents, and that the MC4 receptor may be the receptor subtype mediating such behaviors.

3.2. Regulation of Activity of the HPA Axis

Dysfunction of the HPA axis, caused by continuous exposure to stress, has been reported in patients with major depressive disorder, anorexia nervosa, and post-traumatic stress disorders [30,31]. Several reports suggest that the MC4 receptor may play an important role in regulation of the activity of the HPA axis. Intracerebroventricularly administered ACTH(1-24) increases plasma concentrations of ACTH and corticosterone, which is blocked by the selective MC4 receptor antagonist and SHU9119 [32]. The same report indicates that the selective MC3 receptor agonist, Lys-γ_2-MSH, does not increase plasma ACTH and corticosterone levels [32]. This suggests that the MC4 receptor is responsible for activation of the HPA axis activity by melanocortins. Intracerebroventricular injection of MT II has also been reported to increase plasma corticosterone levels, which are attenuated by the selective MC4 receptor antagonist HS014 [33]. Moreover, local injection of the α-MSH analogue [Nle^4,D-Phe^7]α-MSH into the PVN increases plasma concentrations of ACTH and corticosterone [34]. Parvocellular division of the PVN, which is linked to activation of the HPA axis, expresses only the MC4 receptor, but not the MC3 receptor [21,22], lending support to the hypothesis that MC4 receptor stimulation produces stress-related responses including activation of the HPA axis.

The involvement of the CRF system, the primary hypothalamic factor driving stress-induced ACTH secretion from the anterior pituitary [35], in regulation of the HPA axis mediated through the MC4 receptor has been suggested. First, the MT II-induced increase in plasma corticosterone was attenuated by the nonselective CRF receptor antagonist α-helical CRF(9-41) [33], while α-MSH-increased plasma corticosterone levels are attenuated by anti-CRF antibodies [36]. Second, anatomical interactions have been reported between melanocortinergic system and CRF in the PVN of the hypothalamus. CRF neurons in the PVN are innervated by the α-MSH neuronal terminal [37], and MC4 receptor mRNA is found in the dorsal medial parvocellular part of the PVN [22], which gives rise to most of the CRF fibers that project to the external zone of the median eminence. Moreover, in rats, a subpopulation of CRF-containing neurons (up to 33%) in the PVN contains the MC4 receptor mRNA [33], although a report indicates that MC4 receptor-expressing cells in mice do not express CRF mRNA [38]. Third, functional interactions between the melanocortinergic system and CRF in the PVN have been suggested. Intracerebroventricular injections of α-MSH or MT II increase CRF gene expression in the PVN [33,39], and central administration of α-MSH activates CRF neurons in the medial parvocellular part of the PVN, as assessed by induction of phosphorylated cAMP response element-binding protein, a neuronal activation marker [39]. Moreover, it has also been reported that α-MSH increases release of CRF from hypothalamic explants [34]. Taken together, these findings suggest that the MC4 receptor activates activity of the HPA axis by stimulating the CRF expression and release in the PVN of the hypothalamus. Additionally, it has also been reported that α-MSH increases AVP release from hypothalamic explants [34]. Given that AVP, along with CRF plays a pivotal role in regulation of the HPA axis activity [40] and that the MC4 receptor is expressed in the parvocellular and magnocellular of the PVN where AVP is synthesized and released, AVP may be involved in MC4 receptor-mediated regulation of HPA axis activity.

3.3. Stress-Related Changes in Levels of Expression

Exposure to stress alters the levels at which POMC and the MC4 receptor are expressed. A report indicates that POMC mRNA levels in the arcuate nucleus of the hypothalamus, a major source of melanocortins, is increased by 1 h restraint stress [41], although another study indicates the contradictory finding that repeated immobility stress reduces expression levels of POMC mRNA in the arcuate nucleus [42]. In the previous study, we observed that electrical foot shock stress increased levels of expression of both POMC mRNA and the MC4 receptor mRNA in the hypothalamus [43]. Moreover, it has been reported that adrenalectomy positively [44] or negatively [45] regulates POMC mRNA levels in the arcuate nucleus, that most nuclei of POMC neurons in the arcuate nucleus exhibit glucocorticoid receptor immunoreactivity [45]. Therefore, the levels at which POMC mRNA is expressed in the arcuate nucleus appear to be regulated by feedback mechanisms following stress exposure.

We have also reported that electrical shock stress increases levels of expression of both POMC mRNA and MC4 receptor mRNA in the amygdala, which coincides with increased levels of CRF [43]. The amygdala plays a role in orchestrating various aspects of emotional output and fear-related behaviors [46]. Given that stimulation of melanocortin receptors in the amygdala produces anxiety-like behavior [47], and that the MC4 receptor is widely distributed in the amygdala [21,22], stress-related changes in the expression of MC4 receptor mRNA in the amygdala has significant implications for stress-related behavioral changes.

3.4. Involvement in Anxiety-Like Behavior

Several lines of evidence show that melanocortins induce anxiogenic-like behavior in rodents. As observed with CRF, centrally administered α-MSH or ACTH(1-24) increases isolation-induced distress vocalization in domestic chicks, which presumably represents fear/anxiety [48,49]. It has been reported that intracerebroventricular injection of α-MSH or ACTH(1-24) reduces punished licking times in the rat Vogel conflict test [50]. Consistent with these findings, we reported that intracerebroventricular injection of MT II, an MC4 receptor agonist, significantly reduces social interaction times among unfamiliar paired rats and number of licks in the rat Vogel conflict test, both of which suggest that stimulation of the MC4 receptor produces anxiety-like behavior [29,51] (Fig. (1)).

Some brain regions have been identified as potential sites where melanocortins act to give rise to anxiogenic-like behavior. Injections of α-MSH into the ventromedial nucleus have been reported to stimulate aggressive behavior [52]. The same study indicates that injections of α-MSH into the medial preoptic area reduce exploratory behavior in the elevated plus-maze test, indicating anxiogenic-like effect [52]. Moreover, injections of ACTH(4-10) into the septum decrease time engaged in social interaction, while increasing aggression [53]. Recently, Kokare *et al.* [47] have reported that intra-amygdala administration of α-MSH significantly decrease the proportion of time spent in open arms in the elevated plus-maze.

Although the neuronal mechanisms underlying the anxiogenic-like effects of melanocortins are not fully understood, the involvement of GABAergic transmission has been suggested. It has been reported that diazepam and muscimol attenuate anxiety-like behavior in the elevated plus-maze task induced by α-MSH, while bicuculline, a $GABA_A$ receptor antagonist, enhances the anxiogenic effects of α-MSH [54]. Thus, α-MSH may exert negative influence on GABAergic activity. GABA and clonazepam also reportedly inhibit high K^+-evoked release of α-MSH from hypothalamic slices [55]. In contrast, as in the case of regulation of HPA axis activity, the involvement of CRF activation in anxiogenic effects has been suggested, since increases in both corticosterone levels and anxiety-like behaviors induced by α-MSH are attenuated by intracerebroventricular injection of anti-CRF antibodies [36].

4. ANTIDEPRESSANT AND ANXIOLYTIC EFFECTS OF THE MC4 RECEPTOR ANTAGONISTS

4.1. Antidepressant-Like Activity

We synthesized selective MC4 receptor antagonists such as (Ac-D-2Nal-Arg-2Nal-NH_2 (MCL0020)) [29] and (1-[(S)-2-(4-fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine (MCL0129)) [56] and an MC4 receptor antagonist with serotonin transport inhibitory activity (1-[2-(4-fluorophenyl)-2-(4-methylpiperazin-1-yl)ethyl]-4-[4-(1-naphthyl)butyl]piperazine (MCL0042)) [57] (Fig. (2)), and investigated their antidepressant and anxiolytic effects in several rodent models of depression and anxiety (Figs. (3) and (4)).

MCL0129 significantly shortened immobility time in the rat forced swim test, a behavioral despair model predictive of clinical efficacy, indicating antidepressant-like potential (Fig. (3a)). The effects of MCL0129 in the forced swim test were assessed by a

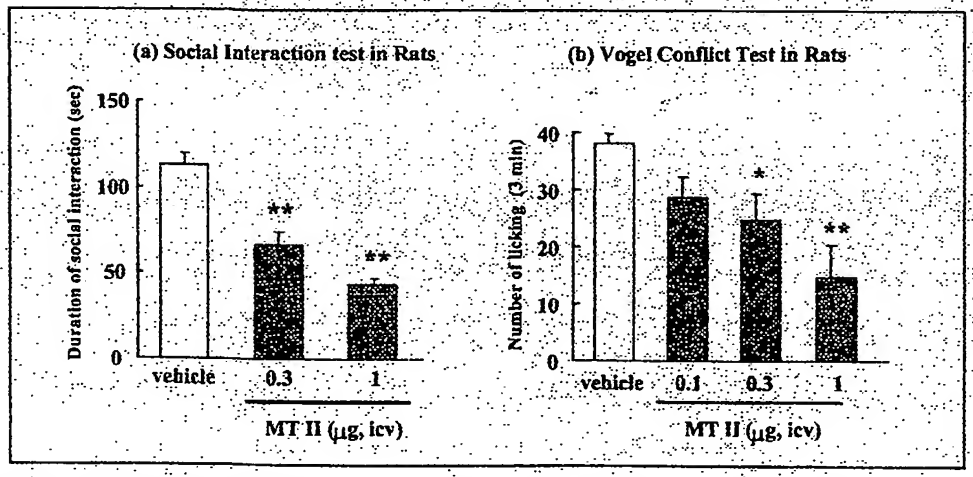
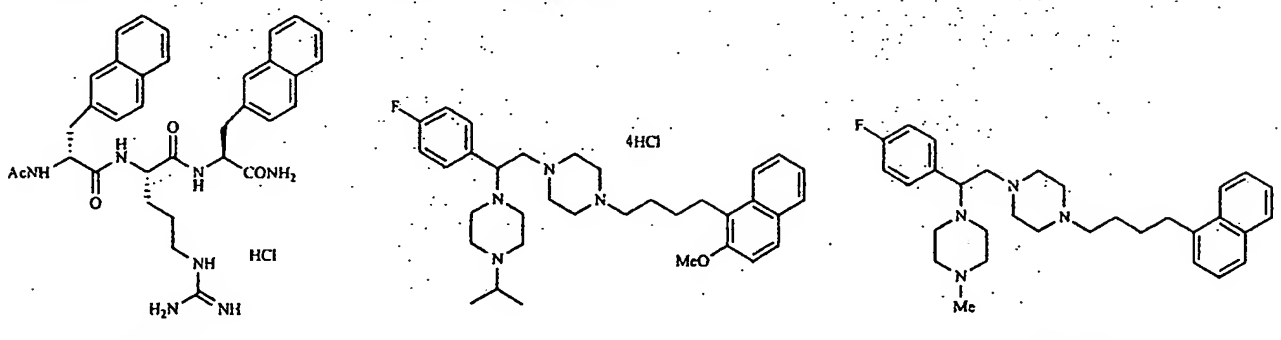


Fig. (1). MC4 receptor agonist-induced anxiety-like behavior in (a) the social interation test and (b) Vogel conflict test in rats. Data represent mean ± SE. *p<0.05, **p<0.01 versus vehicle (Dunnett's test).



MCL0020 MCL0129 MCL0042

Fig. (2). Chemical structures of MCL0020, MCL0129 and MCL0042.

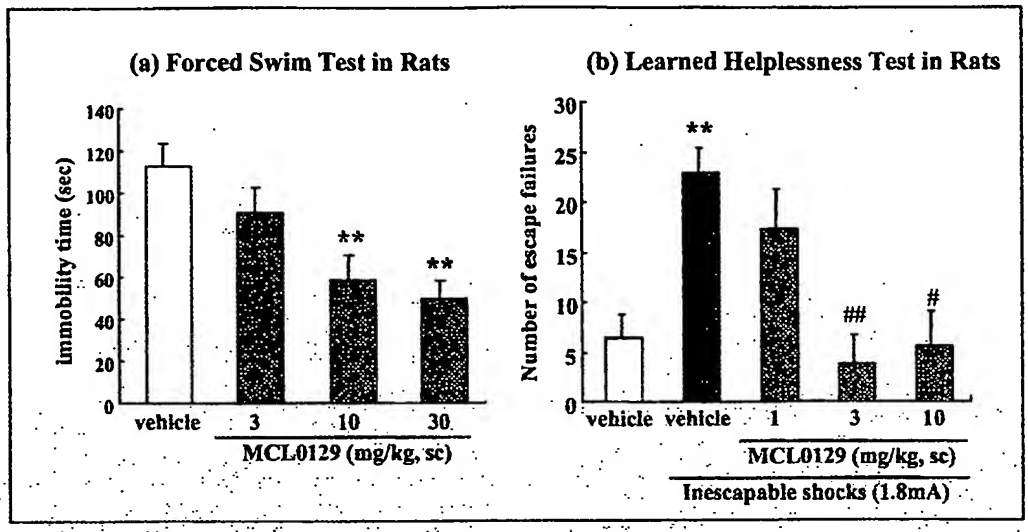


Fig. (3). Antidepressant effects of MCL0129 in (a) forced swim test and (b) learned helplessness test in rats.
Data represent mean ± SE. **p<0.01 versus vehicle (Dunnett's test), #p<0.05, ##p<0.01 versus learned helplessness group (Dunnett's test).

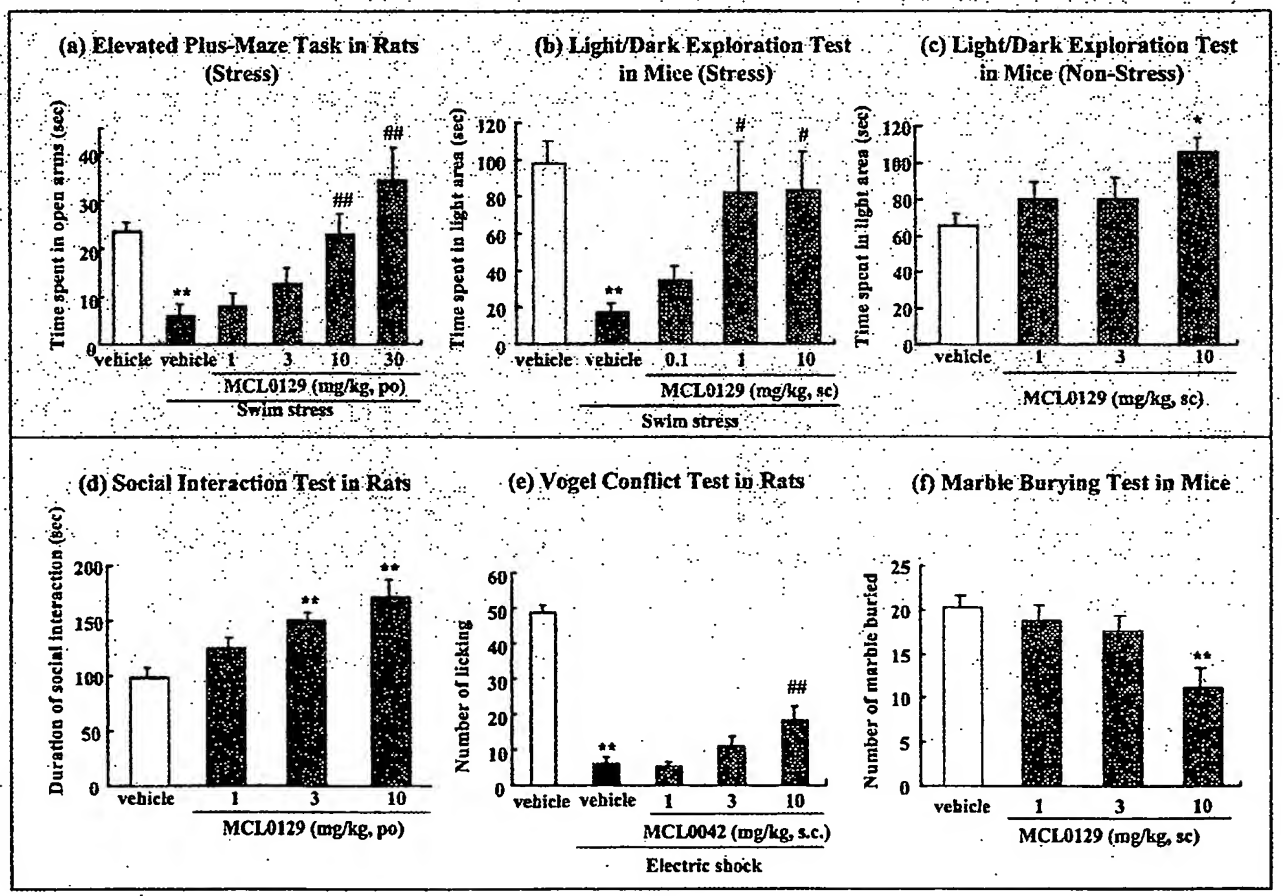


Fig. (4). Anxiolytic effects of MC4 receptor antagonists in various anxiety models.
(a)elevated plus-maze task in rats following swim stress; (b) light/dark exploration test in mice following swimming; (c) light/dark exploration test in mice (non-stress); (d) social interaction test in rats; (e) Vogel conflict test in rats; (f) marble burying test in mice. Data represent mean ± SE. *p<0.05, **p<0.01 versus vehicle (Dunnett's test), #p<0.05, ##p<0.01 versus stress group or electrical shock vehicle group (Dunnett's test).

behavioral sampling method in which test behaviors were classified into three types - immobility, swimming, and climbing. MCL0129 increased swimming behavior without affecting climbing behavior. It has been suggested that in this behavioral scoring method, compounds that potentiate serotonergic transmission may increase swimming scores, whereas drugs that act on noradrenaline

transmission may increase climbing scores [58]. Thus, behavioral patterns induced by MCL0129 are similar to those observed with agents that potentiate serotonergic transmission, such as selective serotonin reuptake inhibitors (SSRIs). Both MC4 receptor mRNA and POMC-imminoreactive axons are present in the dorsal raphe nucleus [21, 59], and we reported that MC4 receptor stimulation modulates neural activity of the dorsal raphe nucleus serotonin neuron [24]. Therefore, an MC4 receptor antagonist may exert antidepressant effects in part by modulating serotonergic neurotransmission.

MCL0129 ameliorated escape deficit in the learned helplessness model in rats, indicating antidepressant effects (Fig. (3b)). Interestingly, in this model, MCL0129 exerted antidepressant effects after acute administration. Both imipramine and fluvoxamine in this model have been reported to show antidepressant effects following chronic treatment, but not single treatment, under the same conditions [60]. This model may reflect the time course of the therapeutic action of antidepressants. In this respect, MC4 receptor antagonists may have a rapid onset of action, which should be beneficial in clinical settings, in light of the lag of several weeks required by current antidepressant medications to exhibit their effects. We have reported that acute treatments of CRF_1 receptor antagonists also exert antidepressant effects in this model [61,62]. Apparently, the antidepressant effects of stress-related peptides antagonists such as MC4 receptor and CRF_1 receptor antagonists in the learned helplessness test may be mediated through mechanisms other than ones that are serotonergic-dependent.

4.2. Anxiolytic-Like activity

Exposure to swim stress has been reported to result in anxiety-like behavior both in the light/dark exploration test (reduction in time spent in the light area) and in the elevated plus-maze test (reduction in time spent in open arms). MCL0129 attenuated swim stress-induced anxiety-like behavior in both paradigms (Fig. (4a,b)). In addition, intracereberoventricular administration of MCL0020 attenuated swim stress-induced anxiety-like behavior in the light/dark exploration test in mice. Thus, MC4 receptor antagonists may reverse behavioral changes induced by stress exposure; conversely, stimulation of the MC4 receptor may be responsible for these behavioral abnormalities. In contrast, MCL0129 exhibited anxiolytic effect in the light/dark exploration test in nonstressed mice (Fig. (4c)), but this effect was weaker than that observed in stressed mice. This trend is consistent with results obtained with other stress-related peptides antagonists such as the CRF_1 receptor antagonist [63] and the V_{1b} receptor antagonists [64], reflecting the etiology of stress-related disorders such as depression and anxiety. We recently reported that MCL0129 significantly prolongs the social interactions of paired unfamiliar rats, indicating anxiolytic effects [51] (Fig. (4d)). It should be mentioned that the social interaction test is also an anxiety model that includes a stress component, since it has been reported that increased plasma ACTH is induced by introducing an unfamiliar pair of rats to a neutral territory [65], and CRF_1 antagonists, whose anxiolytic effects are more prominent in highly stressful conditions, effectively increase social interaction in this paradigm [66].

We recently reported that MCL0042 exhibited anxiolytic effects in the Vogel conflict test in rats, a test including a conflict component (Fig. (4e)). The involvement of the MC4 receptor in the conflict drinking test has been suggested. We reported that intracerebroventricular administration of α-MSH and MT II reduces the number of licking periods in the Vogel test on rats [29], an observation that suggest that the MC4 receptor is involved in the expressing anxiogenic-like behavior in this model. Although MCL0042 shows both MC4 receptor antagonism and serotonin transport inhibition, MC4 receptor blockades appear to be involved in the anxiolytic effects of MCL0042 in the Vogel conflict test, since reports indicate that SSRIs are ineffective in this model [67].

MCL0129 significantly reduced the number of marble buried in mouse marble burying test (Fig. (4f)). Marble-burying behavior is regarded as an animal model predictive of obsessive-compulsive disorder (OCD), based on findings indicating no change in the intensity of marble-burying during repeated testing (this is considered compulsive behavior) as well as observations that SSRIs, now recognized as effective in treating OCD, are effective in this model [68]. This effect is of interest in view of the increasing usefulness of SSRIs in treating subjects with OCD [69].

CONCLUDING REMARKS

It has long been known that brain melanocortinergic systems are involved in a wide range of physiological functions, including feeding behavior, stress response, memory and/or learning, and pain perception, although the receptor subtypes involved in each

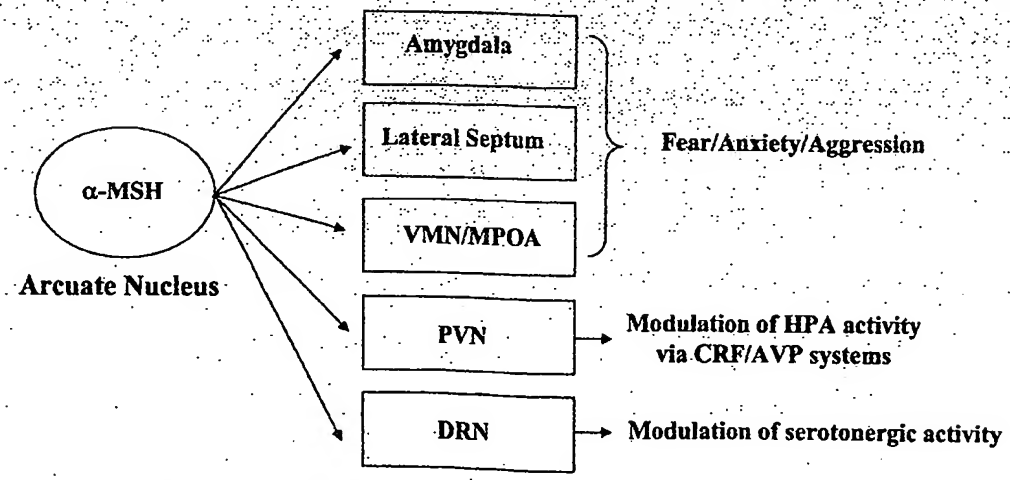


Fig. (5). Neuronal mechanisms underlying stress-related abnormalities mediated through the MC4 receptor.
α-MSH-containing fibers, produced mainly in the arcuate nucleus of the hypothalamus, project to a wide range of brain regions within the hypothalamus and limbic system. α-MSH produces fear and anxiety-like behaviors and increases aggression by stimulating MC4 receptors in the amygdala, lateral septum nucleus, and VMN and MPOA of the hypothalamus. α-MSH activates activity of the HPA axis through MC4 receptor-mediated increased expression and secretion of CRF and/or AVP in the PVN of the hypothalamus. Stimulation of the MC4 receptor in the DRN modulates serotonergic neuronal activity.
VMN; ventromedial nucleus of the hypothalamus, MPOA; medial preoptic area of the hypothalamus, PVN; paraventricular nucleus of the hypothalamus, DRN; dorsal raphe nucleus.

function had yet to be elucidated. Since the cloning in 1993 of MC4 receptor cDNA and identification of MC4 receptor as a main receptor subtype for melanocortins in the brain, the physiological roles of the MC4 receptor have been delineated in a series of studies of receptor distribution and by investigations of phenotypes of genetically engineered animals. Moreover, the development of nonpeptidic MC4 receptor antagonists has provided useful tools for investigating roles of the MC4 receptor. These studies have suggested that the MC4 receptor plays important roles in regulation of feeding and energy balance, and pharmaceutical industry has launched to develop nonpeptidic MC4 receptor agonists for the treatment of obesity.

The identification of the MC4 receptor also implicates the MC4 receptor in stress responses induced by melanocortins. Indeed, an MC4 receptor agonist MT II produces anxiety-like behaviors and functional abnormalities resembling response to stress exposure in rodents, and some of the neuronal mechanisms underlying stress-related responses mediated through the MC4 receptor have been delineated (Fig. (5)). Recent studies using peptidemimetic and nonpeptidic MC4 receptor antagonists have shown that blockades of the MC4 receptor result in antidepressant and anxiolytic effects in several animal models of depression and anxiety. Interestingly, like other antagonists of stress-related peptide receptors such as CRF_1 and V_{1b} receptors, the MC4 receptor antagonist is more effective in high stress conditions than in low stress conditions, consistent with the hypothesis that the MC4 receptor mediates stress response. These findings suggest that MC4 receptor antagonists may be useful for the treatment of stress-related disorders such as depression and anxiety. Whether the data from preclinical studies involving rodents can be extrapolated to humans remains to be determined.

REFERENCES

[1] Fan, W.; Boston, B.A.; Kesterson, R.A.; Hruby, V.J.; Cone, R.D. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 1997, *385*, 165-168.

[2] Seeley, R.J.; Drazen, D.L.; Clegg, D.J. The critical role of the melanocortin system in the control of energy balance. *Annu. Rev. Nutr.* 2004, *24*, 133-149.

[3] Murphy, M.T.; Richards, D.B.; Lipton, J.M. Antipyretic potency of centrally administered alpha-melanocyte stimulating hormone. *Science* 1983, *221*, 192-193.

[4] Vrinten, D.H.; Gispen, W.H.; Groen, G.J.; Adan, R.A. Antagonism of the melanocortin system reduces cold and mechanical allodynia in mononeuropathic rats. *J. Neurosci.* 2000, *20*, 8131-8137.

[5] Ceriani, G.; Macaluso, A.; Catania, A.; Lipton, J.M. Central neurogenic antiinflammatory action of alpha-MSH: modulation of peripheral inflammation induced by cytokines and other mediators of inflammation. *Neuroendocrinology* 1994, *59*, 138-143.

[6] Thody, A.J. alpha-MSH and the regulation of melanocyte function. *Ann. N.Y. Acad. Sci.* 1999, *885*, 217-229.

[7] Gee, C.E.; Chen, C.L.; Roberts, J.L.; Thompson, R.; Watson, S.J. Identification of proopiomelanocortin neurones in rat hypothalamus by in situ cDNA-mRNA hybridization. *Nature* 1983, *306*, 374-376.

[8] Bagnol, D.; Lu, X.Y.; Kaelin, C.B.; Day, H.E.; Ollmann, M.; Gantz, I.; Akil, H.; Barsh, G.S.; Watson, S.J. Anatomy of an endogenous antagonist: relationship between Agouti-related protein and proopiomelanocortin in brain. *J. Neurosci.* 1999, *19*, RC26 1-7.

[9] Kiss, J.Z.; Cassell, M.D.; Palkovits, M. Analysis of the ACTH/beta-End/alpha-MSH-immunoreactive afferent input to the hypothalamic paraventricular nucleus of rat. *Brain Res.* 1984, *324*, 91-99.

[10] Gray, T.S.; Cassell, M.D.; Kiss, J.Z. Distribution of pro-opiomelanocortin-derived peptides and enkephalins in the rat central nucleus of the amygdala. *Brain Res.* 1984, *306*, 354-358.

[11] Lu, D.; Willard, D.; Patel, I.R.; Kadwell, S.; Overton, L.; Kost, T.; Luther, M.; Chen, W.; Woychik, R.P.; Wilkison, W.O.; Cone, R.D. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* 1994, *371*, 799-802.

[12] Ollmann, M.M.; Wilson, B.D.; Yang, Y.K.; Kerns, J.A.; Chen, Y.; Gantz, I.; Barsh, G.S. Antagonism of central melanocortin receptors *in vitro* and *in vivo* by agouti-related protein. *Science* 1997, *278*, 135-138.

[13] Wikberg, J.E. Melanocortin receptors: perspectives for novel drugs. *Eur. J. Pharmacol.* 1999, *375*, 295-310.

[14] Mountjoy, K.G.; Robbins, L.S.; Mortrud, M.T.; Cone, R.D. The cloning of a family of genes that encode the melanocortin receptors. *Science* 1992, *257*, 1248-1251.

[15] Gantz, I.; Konda, Y.; Tashiro, T.; Shimoto, Y.; Miwa, H.; Munzert, G.; Watson, S.J.; DelValle, J.; Yamada, T. Molecular cloning of a novel melanocortin receptor. *J. Biol. Chem.* 1993, *268*, 8246-8250.

[16] Catania, A.; Gatti, S.; Colombo, G.; Lipton, J.M. Targeting melanocortin receptors as a novel strategy to control inflammation. *Pharmacol. Rev.* 2004, *56*, 1-29.

[17] Roselli-Rehfuss, L.; Mountjoy, K.G.; Robbins, L.S.; Mortrud, M.T.; Low, M.J.; Tatro, J.B.; Entwistle, M.L.; Simerly, R.B.; Cone, R.D. Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc. Natl. Acad. Sci. USA* 1993, *90*, 8856-8860.

[18] Butler, A.A.; Kesterson, R.A.; Khong, K.; Cullen, M.J.; Pelleymounter, M.A.; Dekoning, J.; Baetscher, M.; Cone, R.D. A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* 2000, *141*, 3518-3521.

[19] Gantz, I.; Miwa, H.; Konda, Y.; Shimoto, Y.; Tashiro, T.; Watson, S.J.; DelValle, J.; Yamada, T. Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *J. Biol. Chem.* 1993, *268*, 15174-15179.

[20] Alvaro, J.D.; Tatro, J.B.; Quillan, J.M.; Fogliano, M.; Eisenhard, M.; Lerner, M.R.; Nestler, E.J.; Duman, R.S. Morphine down-regulates melanocortin-4 receptor expression in brain regions that mediate opiate addiction. *Mol. Pharmacol.* 1996, *50*, 583-591.

[21] Kishi, T.; Aschkenasi, C.J.; Lee, C.E.; Mountjoy, K.G.; Saper, C.B.; Elmquist, J.K. Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. *J. Comp. Neurol.* 2003, *457*, 213-235.

[22] Mountjoy, K.G.; Mortrud, M.T.; Low, M.J.; Simerly, R.B.; Cone, R.D. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol. Endocrinol.* 1994, *8*, 1298-1308.

[23] Chhajlani, V. Distribution of cDNA for melanocortin receptor subtypes in human tissues. *Biochem. Mol. Biol. Int.* 1996, *38*, 73-80.

[24] Kawashima, N.; Chaki, S.; Okuyama, S. Electrophysiological effects of melanocortin receptor ligands on neuronal activities of monoaminergic neurons in rats. *Neurosci. Lett.* 2003, *353*, 119-122.

[25] Adan, R.A.; Szklarczyk, A.W.; Oosterom, J.; Brakkee, J.H.; Nijenhuis, W.A.; Schaaper, W.M.; Meloen, R.H.; Gispen, W.H. Characterization of melanocortin receptor ligands on cloned brain melanocortin receptors and on grooming behavior in the rat. *Eur. J. Pharmacol.* 1999, *378*, 249-258.

[26] De Barioglio, S.R.; Lezcano, N.; Celis, M.E. Alpha MSH-induced excessive grooming behavior involves a GABAergic mechanism. *Peptides* 1991, *12*, 203-205.

[27] Dunn, A.J.; Green, E.J.; Isaacson, R.L. Intracerebral adrenocorticotropic hormone mediates novelty-induced grooming in the rat. *Science* 1979, *203*, 281-283.

[28] Vergoni, A.V.; Bertolini, A.; Wikberg, J.E.; Schioth, H.B. Selective melanocortin MC4 receptor blockage reduces immobilization stress-induced anorexia in rats. *Eur. J. Pharmacol.* 1999, *369*, 11-15.

[29] Chaki, S.; Ogawa, S.; Toda, Y.; Funakoshi, T.; Okuyama, S. Involvement of the melanocortin MC4 receptor in stress-related behavior in rodents. *Eur. J. Pharmacol.* 2003, *474*, 95-101.

[30] Holsboer, F.; Von Bardeleben, U.; Gerken, A.; Stalla, G.K.; Muller, O.A. Blunted corticotropin and normal cortisol response to human corticotropin-releasing factor in depression. *N. Engl. J. Med.* 1984, *311*, 1127-1133.

[31] Taylor, A.L.; Fishman, L.M. Corticotropin-releasing hormone. *N. Engl. J. Med.* 1988, *319*, 213-222.

[32] Von Frijtag, J.C.; Croiset, G.; Gispen, W.H.; Adan, R.A.; Wiegant, V.M. The role of central melanocortin receptors in the activation of the hypothalamus-pituitary-adrenal-axis and the induction of excessive grooming. *Br. J. Pharmacol.* 1998, *123*, 1503-1508.

[33] Lu, X.Y.; Barsh, G.S.; Akil, H.; Watson, S.J. Interaction between alpha-melanocyte-stimulating hormone and corticotropin-releasing hormone in the regulation of feeding and hypothalamo-pituitary-adrenal responses. *J. Neurosci.* 2003, *23*, 7863-7872.

[34] Dhillo, W.S.; Small, C.J.; Seal, L.J.; Kim, M.S.; Stanley, S.A.; Murphy, K.G.; Ghatei, M.A.; Bloom, S.R. The hypothalamic melanocortin system stimulates the hypothalamo-pituitary-adrenal axis *in vitro* and in vivo in male rats. *Neuroendocrinology* 2002, *75*, 209-216.

[35] Rivier, C.L.; Plotsky, P.M. Mediation by corticotropin releasing factor (CRF) of adenohypophysial hormone secretion. *Annu. Rev. Physiol.* 1986, *48*, 475-494.

[36] Vecsernyes, M.; Biro, E.; Gardi, J.; Julesz, J.; Telegdy, G. Involvement of endogenous corticotropin-releasing factor in mediation of neuroendocrine and behavioral effects to alpha-melanocyte-stimulating hormone. *Endocr. Res.* 2000, *26*, 347-356.

[37] Liposits, Z.; Sievers, L.; Paull, W.K. Neuropeptide-Y and ACTH-immunoreactive innervation of corticotropin releasing factor (CRF)-synthesizing neurons in the hypothalamus of the rat. An immunocytochemical analysis at the light and electron microscopic levels. *Histochemistry* 1988, *88*, 227-234.

[38] Liu, H.; Kishi, T.; Roseberry, A.G.; Cai, X.; Lee, C.E.; Montez, J.M.; Friedman, J.M.; Elmquist, J.K. Transgenic mice expressing green fluorescent protein under the control of the melanocortin-4 receptor promoter. *J. Neurosci.* 2003, *23*, 7143-7154.

[39] Sarkar, S.; Legradi, G.; Lechan, R.M. Intracerebroventricular administration of alpha-melanocyte stimulating hormone increases phosphorylation of

CREB in TRH- and CRH-producing neurons of the hypothalamic paraventricular nucleus. *Brain Res.* 2002, *945*, 50-59.

[40] Aguilera, G.; Rabadan-Diehl, C. Vasopressinergic regulation of the hypothalamic-pituitary-adrenal axis: implications for stress adaptation. *Regul. Pept.* 2000, *96*, 23-29.

[41] Larsen, P.J.; Mau, S.E. Effect of acute stress on the expression of hypothalamic messenger ribonucleic acids encoding the endogenous opioid precursors preproenkephalin A and proopiomelanocortin. *Peptides* 1994, *15*, 783-790.

[42] Makino, S.; Asaba, K.; Nishiyama, M.; Hashimoto, K. Decreased type 2 corticotropin-releasing hormone receptor mRNA expression in the ventromedial hypothalamus during repeated immobilization stress. *Neuroendocrinology* 1999, *70*, 160-167.

[43] Yamano, Y.; Yoshioka, M.; Toda, Y.; Oshida, Y.; Chaki, S.; Hamamoto, K.; Morishima, I. Regulation of CRF, POMC and MC4R gene expression after electrical foot shock stress in the rat amygdala and hypothalamus. *J. Vet. Med. Sci.* 2004, *66*, 1323-1327.

[44] Meyer, J.S.; Micco, D.J.; Stephenson, B.S.; Krey, L.C.; McEwen, B.S. Subcutaneous implantation method for chronic glucocorticoid replacement therapy. *Physiol. Behav.* 1979, *22*, 867-870.

[45] Ozawa, H.; Lion, J.; Xie, C.X.; Nishi, M.; Steinbusch, H.; Kawata, M. Down-regulation of ACTH and glucocorticoid receptor immunoreactivity in hypothalamic arcuate neurons after adrenalectomy in the rat. *Neuroreport* 1999, *10*, 1571-1575.

[46] Davis, M.; Shi, C. The extended amygdala: are the central nucleus of the amygdala and the bed nucleus of the stria terminalis differentially involved in fear versus anxiety? *Ann. N.Y. Acad. Sci.* 1999, *877*, 281-291.

[47] Kokare, D.M.; Dandekar, M.P.; Chopde, C.T.; Subhedar, N. Interaction between neuropeptide Y and alpha-melanocyte stimulating hormone in amygdala regulates anxiety in rats. *Brain Res.* 2005, *1043*, 107-114.

[48] Panksepp, J.; Abbott, B.B. Modulation of separation distress by alpha-MSH. *Peptides* 1990, *11*, 647-653.

[49] Panksepp, J.; Normansell, L. Effects of ACTH(1-24) and ACTH/MSH(4-10) on isolation-induced distress vocalization in domestic chicks. *Peptides* 1990, *11*, 915-919.

[50] Corda, M.G.; Orlandi, M.; Frana, W. Proconflict effect of ACTH1-24: interaction with benzodiazepines. *Pharmacol. Biochem. Behav.* 1990, *36*, 631-634.

[51] Shimazaki, T.; Chaki, S. Anxiolytic-like effect of a selective and non-peptidergic melanocortin 4 receptor antagonist, MCL0129, in a social interaction test. *Pharmacol. Biochem. Behav.* 2005, *80*, 395-400.

[52] Gonzalez, M.I.; Vaziri, S.; Wilson, C.A. Behavioral effects of alpha-MSH and MCH after central administration in the female rat. *Peptides* 1996, *17*, 171-177.

[53] Clarke, A.; File, S.E. Social and exploratory behaviour in the rat after septal administration of ORG 2766 and ACTH4-10. *Psychoneuroendocrinology* 1983, *8*, 343-350.

[54] Rao, T.L.; Kokare, D.M.; Sarkar, S.; Khisti, R.T.; Chopde, C.T.; Subhedar, N. GABAergic agents prevent alpha-melanocyte stimulating hormone induced anxiety and anorexia in rats. *Pharmacol. Biochem. Behav.* 2003, *76*, 417-423.

[55] Blasquez, C.; Jegou, S.; Tranchand-Bunel, D.; Delbende, C.; Braquet, P.; Vaudry, H. Central-type benzodiazepines inhibit release of alpha-melanocyte-stimulating hormone from the rat hypothalamus. *Neuroscience* 1991, *42*, 509-516.

[56] Chaki, S.; Hirota, S.; Funakoshi, T.; Suzuki, Y.; Suetake, S.; Okubo, T.; Ishii, T.; Nakazato, A.; Okuyama, S. Anxiolytic-like and antidepressant-like activities of MCL0129 (1-[(S)-2-(4-fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine), a novel and potent nonpeptide antagonist of the melanocortin-4 receptor. *J. Pharmacol. Exp. Ther.* 2003, *304*, 818-826.

[57] Chaki, S.; Oshida, Y.; Ogawa, S.; Funakoshi, T.; Shimazaki, T.; Okubo, T.; Nakazato, A.; Okuyama, S. MCL0042: a nonpeptidic MC4 receptor antagonist and serotonin reuptake inhibitor with anxiolytic- and antidepressant-like activity. *Pharmacol. Biochem. Behav.* 2005, *82*, 621-626

[58] Cryan, J.F.; Markou, A.; Lucki, I. Assessing antidepressant activity in rodents: recent developments and future needs. *Trends Pharmacol. Sci.* 2002, *23*, 238-245.

[59] Zheng, Z.; Leger, L.; Cespuglio, R.; Jouvet, M. Distribution of the pro-opiomelanocortin-immunoreactive axons in relation to the serotoninergic neurons in the dorsal raphe nucleus of the rat. *Neurosci. Lett.* 1991, *130*, 17-21.

[60] Takamori, K.; Yoshida, S.; Okuyama, S. Repeated treatment with imipramine, fluvoxamine and tranylcypromine decreases the number of escape failures by activating dopaminergic systems in a rat learned helplessness test. *Life Sci.* 2001, *69*, 1919-1926.

[61] Chaki, S.; Nakazato, A.; Kennis, L.; Nakamura, M.; Mackie, C.; Sugiura, M.; Vinken, P.; Ashton, D.; Langlois, X.; Steckler, T. Anxiolytic- and antidepressant-like profile of a new CRF1 receptor antagonist, R278995/CRA0450. *Eur. J. Pharmacol.* 2004, *485*, 145-158.

[62] Takamori, K.; Kawashima, N.; Chaki, S.; Nakazato, A.; Kameo, K. Involvement of corticotropin-releasing factor subtype 1 receptor in the acquisition phase of learned helplessness in rats. *Life Sci.* 2001, *69*, 1241-1248.

[63] Okuyama, S.; Chaki, S.; Kawashima, N.; Suzuki, Y.; Ogawa, S.; Nakazato, A.; Kumagai, T.; Okubo, T.; Tomisawa, K. Receptor binding, behavioral, and electrophysiological profiles of nonpeptide corticotropin-releasing factor subtype 1 receptor antagonists CRA1000 and CRA1001. *J. Pharmacol. Exp. Ther.* 1999, *289*, 926-935.

[64] Griebel, G.; Simiand, J.; Serradeil-Le Gal, C.; Wagnon, J.; Pascal, M.; Scatton, B.; Maffrand, J.P.; Soubrie, P. Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. *Proc. Natl. Acad. Sci. USA* 2002, *99*, 6370-6375.

[65] File, S.E.; Seth, P. A review of 25 years of the social interaction test. *Eur. J. Pharmacol.* 2003, *463*, 35-53.

[66] Millan, M.J.; Brocco, M.; Gobert, A.; Dekeyne, A. Anxiolytic properties of the selective, non-peptidergic CRF(1) antagonists, CP154,526 and DMP695: a comparison to other classes of anxiolytic agent. *Neuropsychopharmacology* 2001, *25*, 585-600.

[67] Borsini, F.; Podhorna, J.; Marazziti, D. Do animal models of anxiety predict anxiolytic-like effects of antidepressants? *Psychopharmacology (Berl)* 2002, *163*, 121-141.

[68] Njung'e, K.; Handley, S.L. Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. *Br. J. Pharmacol.* 1991, *104*, 105-112.

[69] Pigott, T.A.; Seay, S.M. A review of the efficacy of selective serotonin reuptake inhibitors in obsessive-compulsive disorder. *J. Clin. Psychiatry* 1999, *60*, 101-106.

Blockade of melanocortin transmission inhibits cocaine reward

Richard Hsu,[1] Jane R. Taylor,[1] Samuel S. Newton,[1] John D. Alvaro,[1] Colin Haile,[1] G. Han,[2] Victor J. Hruby,[2] Eric J. Nestler[3] and Ronald S. Duman[1]
[1]Laboratory of Molecular Psychiatry, Center for Genes and Behaviour, Departments of Psychiatry and Pharmacology, Yale University School of Medicine, New Haven, CT, USA
[2]University of Arizona, Tucson, AZ, USA
[3]The University of Texas South-western Medical Center, Dallas, TX, USA

Keywords: dopamine, drugs of abuse, motivation, nucleus accumbens, rodent

Abstract

Melanocortins and the melanocortin-4 receptor (MC4-R) are enriched in the nucleus accumbens, a brain region that has been implicated in the rewarding action of cocaine and other drugs of abuse. In the present study we use a number of rat behavioral models to show that infusion of a melanocortin peptide antagonist into the nucleus accumbens blocks the reinforcing, incentive motivational, and locomotor sensitizing effects of cocaine. We also show that locomotor responses to repeated cocaine exposure are completely blocked in MC4-R null mutant mice and reduced in Agouti mice that overexpress an endogenous inhibitor of melanocortins in the brain. The results also demonstrate that cocaine administration increases the expression of MC4-R in the nucleus accumbens and striatum, and that MC4-R is co-localized with prodynorphin in medium spiny neurons in the nucleus accumbens. Together, these findings indicate that the behavioral actions of cocaine are dependent on activation of MC4-R, and suggest that upregulation of this receptor by drug exposure may contribute to sensitization of these behavioral responses. Modulation of cocaine reward is a novel action of the melanocortin–MC4-R system and could be targeted for the development of new medications for cocaine addiction.

Introduction

The melanocortin neuropeptides α, β and γ melanocyte-stimulating hormone (MSH), which are derived from proopiomelanocortin (POMC)-expressing neurons in the arcuate nucleus of the hypothalamus have been implicated in a number of behavioral and neuroendocrine responses, including grooming, thermoregulation and learning (Spruijt *et al.*, 1992; Alvaro *et al.*, 1997). In addition, the melanocortin system is involved in the control of feeding behavior via regulation of the melanocortin-4 receptor (MC4-R) in the hypothalamus (Fan *et al.*, 1997; Huszar *et al.*, 1997). Melanocortin neuropeptides have also been implicated in the actions of opiates, including modulation of opiate tolerance, dependence and withdrawal (Szekaly *et al.*, 1979; Contreras & Takemori, 1984; Alvaro *et al.*, 1997), and we have reported that the expression of the MC4-R is regulated by repeated opiate administration (Alvaro *et al.*, 1996, 2003).

The role of melanocortins in drug-seeking and -taking behavior has not received as much attention. This is surprising given the amount of evidence demonstrating an interaction between the melanocortin system and dopaminergic neurotransmission in regions known to regulate drug reinforcement and motivational processes. Previous studies have demonstrated an enrichment of melanocortin neuropeptides and MC4-R in dopamine-rich brain regions, including nucleus accumbens and dorsal striatum (Jacobowitz & O'Donohue, 1978; Eskay *et al.*, 1979; Mountjoy *et al.*, 1994; Alvaro *et al.*, 1996; Adan & Gispen, 1997). Moreover, functional interactions between melanocortins and dopaminergic neurotransmission have been demonstrated in these brain regions. For example, infusions of a melanocortin agonist into the lateral ventricle or ventral tegmental area increase dopamine release in the striatum and nucleus accumbens (Florijn *et al.*, 1993a; Lindblom *et al.*, 2001). Melanocortin agonist infusions into the nucleus accumbens also increase dopaminergic activity that is associated with increased stereotypic behaviors, such as grooming (Ryan & Isaacson, 1983; Torre & Celis, 1986; Spruijt *et al.*, 1992; Florijn *et al.*, 1993a; Lezcano *et al.*, 1995). In addition, *in vitro* studies demonstrate that α-MSH stimulates cAMP production via interactions with dopamine D1 receptors (Lezcano *et al.*, 1995; Cremer *et al.*, 2000). Activation of dopamine neurotransmission, by administration of either a direct-acting dopamine agonist or an indirect agonist like cocaine, is also reported to upregulate POMC expression in the arcuate nucleus and melanocortin neuropeptide levels in nucleus accumbens (Samyai *et al.*, 1992; Tong & Pelletier, 1992). Finally, one recent study demonstrated that infusion of a melanocortin agonist into the lateral ventricle enhances the effects of amphetamine on lateral hypothalamic self-stimulation (Cabeza de Vaca *et al.*, 2002). Together, these studies indicate that melanocortins enhance dopaminergic neurotransmission, and that dopamine and psychostimulants increase melanocortin function.

Given the localization of melanocortins and MC4-R in brain reward regions and functional interactions with dopamine, the current study

Correspondence: Dr R. S. Duman, 34 Park St, New Haven, CT 06508, USA.
E-mail: ronald.duman@yale.edu

Received 23 July 2004, revised 1 February 2005, accepted 11 February 2005

doi:10.1111/j.1460-9568.2005.04038.x

was undertaken to test the hypothesis that the melanocortin signaling system is necessary for the reinforcing and locomotor activating effects of cocaine. Using a combination of pharmacological and mutant mouse approaches to modulate melanocortin activity, the results of behavioral studies demonstrate that blockade of melanocortins or MC4-R inhibits reinforcing, incentive motivational and locomotor activating responses to cocaine.

Materials and methods

Cocaine self-administration

Rats were used for all behavioral studies, with the exception of the locomotor studies conducted in Agouti with MC4-R null mutant mice as indicated. Intra-accumbens infusions of SHU-9119 or saline vehicle were given via a bilateral indwelling cannulae (23 gauge) using standard stereotaxic procedures. Cannulae placements were confirmed at the conclusion of the experiments by examining the cannulae tracts in collected brains. All animal procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Yale Animal Care and Use Committee. Cocaine self-administration experiments were conducted according to a standard protocol (Piazza *et al.*, 2000). Male Sprague–Dawley rats (300–400 g) were first trained for food self-administration on a fixed-ratio 1 (FR1) schedule until all animals were self-administering 100 pellets of food per session for at least three consecutive days. Animals were administered Equithesin (4.5 mg/kg, i.p.) anesthesia. Cannulae were then surgically implanted bilaterally in the nucleus accumbens for infusions of a SHU-9119, a selective melanocortin receptor antagonist (Hruby *et al.*, 1995), and a catheter was placed in a jugular vein for administration of cocaine. After a 2-week recovery period, rats were trained to lever press for cocaine (0.50 mg/kg/injection) on a FR1 schedule until stable, defined as lever pressing with less than 10% variability on three consecutive days. Rats self-administered an average of 25 mg cocaine, with no subject exhibiting less than 28 lever presses per training session. In the first experiment, the influence of vehicle or different doses of SHU-9119 (0.125, 0.25, 0.50 and 1.00 μg in 0.5 μL per side) infused into the nucleus accumbens 20 min prior to the start of the self-administration session on lever pressing for cocaine was determined. The stereotaxic coordinates for the nucleus accumbens cannula placements were: AP +1.7 from bregma, ML ±1.5 from midline, DV −6.0 from the skull. Each animal was infused with a different dose of SHU-9119 in a randomized order for effects on lever pressing for cocaine. Animals received another dose of SHU-9119, but not until they were returned to stable lever pressing (minimum of 25 lever presses per session) for cocaine for at least 3 days and not more than 5 days. Each animal received a minimum of two infusions but not more than five infusions. In addition, the first three doses (0.125, 0.25 and 0.50 μg) were tested first in randomized order because these doses were determined to be in the effective dose range from preliminary studies. In the second experiment, another group of rats were again trained until stable on 0.50 mg/kg/injection of cocaine. On test day 1 the animals were allowed to self-administer one of three different doses of cocaine (0.125, 0.25, 0.50 mg/kg/injection). On test day 2 the animals were infused with a fixed dose of SHU-9119 (0.25 μg per side), and lever pressing for the same dose of cocaine tested on the previous day was determined. The 0.25-μg dose of SHU-9119 was chosen because the higher doses (0.50 and 1.0 μg) completely blocked lever pressing for cocaine, even at the highest dose of cocaine tested (0.50 mg/kg). Animals were returned to stable lever pressing for cocaine at 0.5 mg/kg/injection of cocaine for at least 3 days and not more than 15 days before they were infused with the same dose of SHU-9119 (0.25 μg) but a different dose of cocaine. Each animal received a total of three infusions of SHU-9119 and was tested on the three different doses of cocaine lever pressing.

Cocaine place conditioning

In this test, animals (rats for this study) repeatedly exposed to cocaine in a distinctive environment will come to spend more time in that environment in the absence of cocaine. This type of drug-induced place conditioning is considered a measure of the rewarding effects of cocaine (Van der Kooy, 1987). Place conditioning was conducted using a three-compartment test as described previously (Carlezon *et al.*, 1998). Pre-test studies demonstrated that there was no baseline bias for a particular chamber. Three different procedures were used to determine the influence of SHU-9119 on conditioning to cocaine when given on each training day or on the test day only, in the absence of cocaine in animals that have been previously paired or not paired with cocaine. For the first procedure, subjects either received an intra-accumbens infusion of SHU-9119 (1.0 μg/0.5 μL) or saline 30 min before each cocaine or saline injection on each of six training days (3 days pairing with cocaine and 3 days pairing with saline), but not on the test day when there was no cocaine administered. In the second procedure subjects were paired with cocaine or saline exactly as described for the first procedure but did not receive infusions of SHU-9119; SHU-9119 was then infused 30 min prior to testing on the test day only, in the absence of cocaine. In the third paradigm, saline or SHU-9119 was infused into the nucleus accumbens prior to pairing with saline for 6 days to determine if the antagonist alone influenced preference on the test day. In all cases conditioning was established on six consecutive days by administering cocaine (15 mg/kg, i.p.) or saline given 5 min before confining the animals to one of the two outside conditioning compartments of the three-compartment chamber for 30 min. On alternative conditioning sessions, subjects were given the other treatment and confined to the opposite compartment after a saline or SHU-9119 infusion. On the test day, subjects were infused with saline, placed in the middle compartment and were subsequently allowed 30 min to freely explore all three compartments of the apparatus.

Responding for conditioned reinforcement: effect of cocaine

Enhanced responding for conditioned reinforcement (CR) is increased by exposure to psychomotor stimulant drugs, effects dependent on dopamine in the nucleus accumbens (Taylor & Robbins, 1984, 1986). The ability of prior cocaine exposure to enhance responding for conditioned reinforcers also has been demonstrated and has been hypothesized to contribute to increases in the incentive salience of reward-related stimuli in addiction (Robinson & Berridge, 1993; Jentsch & Taylor, 1999; Taylor & Horger, 1999). Responding for CR was conducted as previously described (Horger *et al.*, 1999). Thirsty rats were trained to associate a compound (tone + light) stimulus with water reward for 15 days. Animals were otherwise restricted to 30 min access to water in the home cage after the training or test sessions (except during surgery or recovery). Food was available *ad libitum*. On two subsequent test sessions separated by 2 days, they were given cocaine (15 mg/kg) or saline injections 20 min after an intra-accumbens infusion of SHU-9119 (1.0 μg/0.5 μL) and responding for conditioned reinforcement was measured. Responding on one lever resulted in the delivery of the conditioned stimulus (conditioned reinforcer or CR lever), but without the water reinforcement, whereas

responding on the other lever resulted in no CR stimulus presentation (NCR lever).

Locomotor sensitization to cocaine

Locomotor sensitization in rats was conducted as previously described (Carlezon *et al.*, 1998). Subjects were habituated to the test apparatus over three consecutive days. Horizontal locomotor activity was quantified using automated beam crossing. Rats were used for the SHU-9119 infusion studies. Thirty minutes after intra-accumbens SHU-9119 infusions (1.0 μg/0.5 μL) or saline, rats were injected with cocaine (15 mg/kg, i.p.) and placed in the apparatus. This procedure was repeated daily over four or five consecutive days. Locomotor sensitization in mice was conducted as previously described (Hiroi *et al.*, 1997). Two mutant mouse models, Agouti mice and MC4-R null mutant mice, were used to further test the influence of the melanocortin system in the actions of cocaine. Agouti mice display ectopic expression of the agouti peptide, which acts as an antagonist of melanocortin receptors in brain and other tissues, and not just skin where it is normally expressed. Agouti mice (C57BL/6J-A^y, #00021) and C57Bl/6J controls (#000664), both from Jackson Laboratories (Bar Harbor, MA, USA), of the same age (10–12 weeks) and weight (30–35 g) were used for these studies. It is possible that behavioral differences between the Agouti and C57Bl/6J are also influenced by genetic background differences, which are not identical in these two lines. MC4-R is one of the most prevalent melanocortin receptor subtypes expressed in brain, and previous studies have demonstrated that this receptor is expressed in the striatum and nucleus accumbens and is regulated by chronic administration of morphine (Alvaro *et al.*, 1996). MC4-R null mutant mice, on a C57BL/6J background, were kindly provided by the laboratory of Dr Dennis Huszar (Millenium Pharmaceuticals, Boston, MA, USA). Wild type (+/+), heterozygous (+/−) and homozygous (−/−) littermates (10–12 weeks old) were used for locomotor sensitization experiments. Mice were habituated to the test apparatus over three consecutive days, and then received cocaine (10 mg/kg, i.p.) or saline injections for six consecutive days. Horizontal locomotor activity was quantified by automated beam crossing for 60 min each day.

RNAse protection and in situ hybridization analysis

Rats were treated with saline or cocaine (15 mg/kg, twice per day, i.p.) for 14 days, killed by decapitation, and brains were collected 3 h after the last injection. The dorsal and nucleus accumbens were dissected together to provide sufficient tissue and total RNA for the RNase protection assay of MC4-R as described (Alvaro *et al.*, 1996). Briefly, total RNA extracted (30 μg) from dissected tissues is hybridized with a ^{32}P-labeled antisense MC4-R riboprobe in solution overnight. After RNase treatment, the double-stranded 'protected' RNA was isolated by polyacrylamide gel electrophoresis and quantified by densitometry of film autoradiograms. Double *in situ* hybridization was conducted according to a published protocol using a ^{33}P-labeled MC4-R riboprobe and digoxigenin-labeled prodynorphin or proenkephalin riboprobes according to standard procedures (Stone *et al.*, 1999). Color reactions were carried out on the hybridized sections, which were then exposed to Kodak emulsion for 14 days, and developed.

Results

Infusion of a melanocortin antagonist blocks the rewarding and locomotor-activating effects of cocaine in rat

The primary reinforcing effects of cocaine were examined by determining the influence of intra-accumbens infusions of the melanocortin antagonist, SHU-9119, on cocaine self-administration. This synthetic peptide antagonist has approximately 10-fold greater affinity for MC4-R than for MC3-R, the other major melanocortin receptor subtype found in brain (Hruby *et al.*, 1995). Cannulae were surgically placed in the nucleus accumbens and then rats were trained to lever press for cocaine as described in the Materials and methods. Cannula placement was verified by sectioning the brains after the completion of behavioral testing. We found that infusion of SHU-9119 at doses of 0.50 and 1.00 μg per side (in 0.5 μL) into nucleus accumbens completely blocked cocaine self-administration, while lower doses (0.125 and 0.25 μg per side) had no effect (data not shown). Next, we determined the influence of a fixed dose of SHU-9119, 0.25 μg per side, over a dose range of cocaine (0.125, 0.25 and

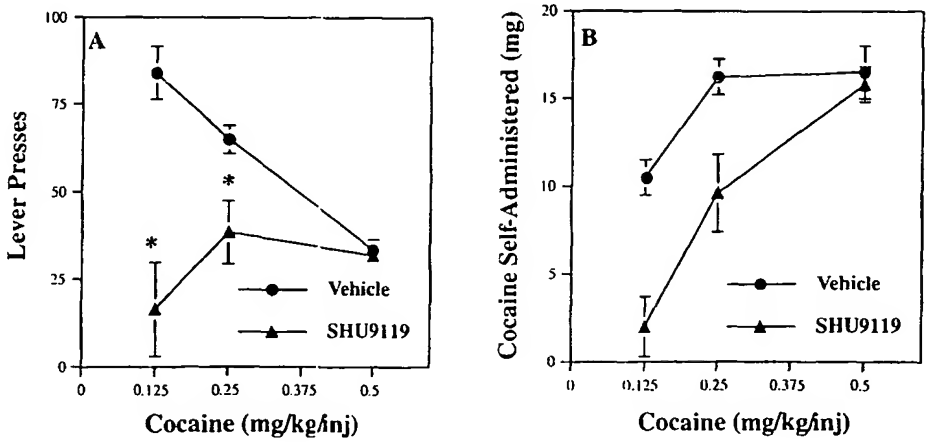


FIG. 1. Cocaine self-administration is blocked by intra-accumbens infusion of a melanocortin antagonist. The effect of direct infusions of SHU-9119 into the nucleus accumbens on lever pressing for cocaine was determined. (A) Rats were trained to lever press for cocaine (0.50 mg/kg/injection) and tested with different doses of cocaine on test day 1 as indicated. Subjects were then tested in the presence of a single dose of SHU-9119 (0.25 μg/side) on test day 2 at the same dose of cocaine from the previous day. The results are expressed as the number of lever presses and are the means ± SEM (n = 3 for the 0.125 dose of cocaine and n = 6 animals for each of the two higher doses of cocaine, 0.25 and 0.50). Repeated-measures ANOVA shows a significant interaction between treatment groups (vehicle vs. SHU-9119) and dose of cocaine ($F_{2,12}$ = 13.99). *Post-hoc* analysis demonstrates that there are significant differences at the two lower doses of cocaine (*P < 0.01). (B) The results are expressed as the total amount of cocaine self-administered at each dose in the absence or presence of SHU-9119.

0.50 mg/kg/injection). This dose of SHU-9119 was chosen so that a dose–response to cocaine could be examined, as preliminary data demonstrated higher doses of SHU-9119 (0.50 and 1.0 μg per side) completely blocked self-administration of 0.50 mg/kg cocaine. SHU-9119 completely blocked level pressing for the lowest dose of cocaine (0.125 mg/kg/injection) and partially, but significantly, blocked the intermediate dose of cocaine (0.25 mg/kg/injection) (Fig. 1A). In contrast, this low dose of SHU-9119 had no effect on lever pressing for the higher cocaine dose (0.5 mg/kg/injection). These data were also plotted as the total amount of cocaine self-administered (Fig. 1B).

To examine the role of the melanocortin system on the conditioned rewarding properties of cocaine, the influence of intra-accumbens infusion of SHU-9119 on cocaine place preference was examined as described in Materials and methods. Three different SHU-9119 infusion schedules were tested to determine the influence of the melanocortin antagonist on conditioning to cocaine during each paired session and on the test day only in the absence of cocaine either with or without prior cocaine pairing (Carlezon *et al.*, 1998). First, SHU-9119 was infused into the nucleus accumbens each day 30 min prior to cocaine (15 mg/kg) pairing with a particular compartment, but not on the test day. Having established an effective dose range for SHU-9119 in the self-administration paradigm, we chose a dose of 1.0 μg/side in 0.5 μL for these studies. This dose of SHU9119 does not influence locomotor activity of rats compared with saline infusions (see locomotor sensitization experiments). Under these conditions, a significant increase in cocaine place conditioning was observed in the vehicle-infused animals (Fig. 2A). In contrast, this rewarding effect of cocaine was changed to conditioned avoidance with infusion of SHU-9119. This suggests that cocaine was made aversive by pre-treatment with the melanocortin receptor antagonist. In the second paradigm, SHU-9119 was infused on the test day only, and not on the previous days when cocaine was administered (Fig. 2B). Infusion of SHU-9119 on the test day also produced conditioned avoidance as was observed in the previous paradigm. Finally, SHU-9119 was infused each day before saline administration (Fig. 2C). Under these conditions, without exposure to cocaine, SHU-9119 did not produce either avoidance or preference to the saline-paired side.

The influence of the melanocortin receptor antagonist on responding for non-drug (i.e. water) reward-related CR was also examined to assess incentive motivation. Intra-accumbens SHU-9119 (1.0 μg per side in 0.5 μL) significantly blocked the ability of cocaine (15 mg/kg) to enhance responding for CR, but did not effect responding on the control or NCR that did not result in presentation of the CR stimulus (Fig. 3A) This suggests that melanocortin receptor antagonism could selectively produce alterations in dopamine-dependent CR mechanisms rather than produce changes related to non-specific motor activation. After saline challenge, intra-accumbens SHU-9119 also significantly attenuated responding on the CR, but not on the control (NCR) lever (Fig. 3B), suggesting that under baseline conditions intra-

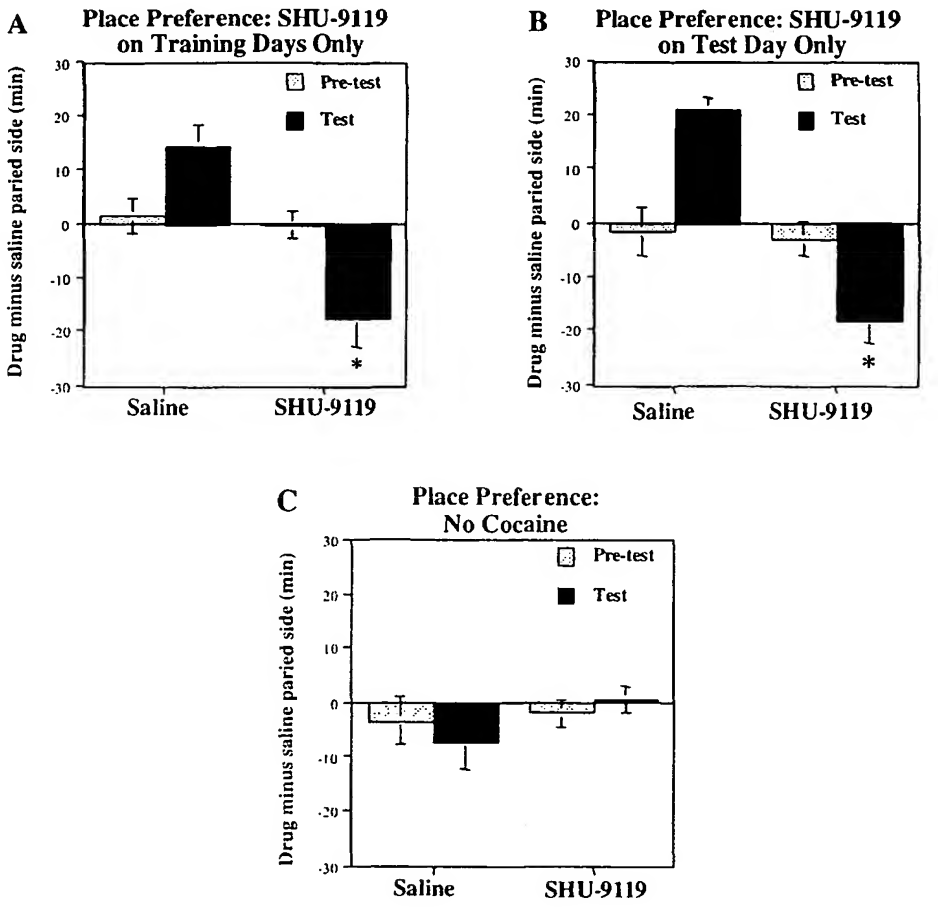


FIG. 2. Cocaine-induced place conditioning is blocked by intra-accumbens infusion of SHU-9119. Rats received intra-accumbens infusions of SHU-9119 (1 μg/0.5 μL) or vehicle according to three different schedules. (A) SHU-9119 was infused each day 30 min before cocaine administration, but not on the test day. (B) SHU-9119 was infused on the test day only. (C) SHU-9119 was infused alone in the absence of cocaine. The results are presented as time spent in the drug-paired side minus time spent in the saline-paired side, and are the mean ± SEM of eight–nine animals per group (A and B) or six–seven per group (C). $^*P < 0.05$ compared with saline-infused control (ANOVA and Fisher's *post-hoc* test).

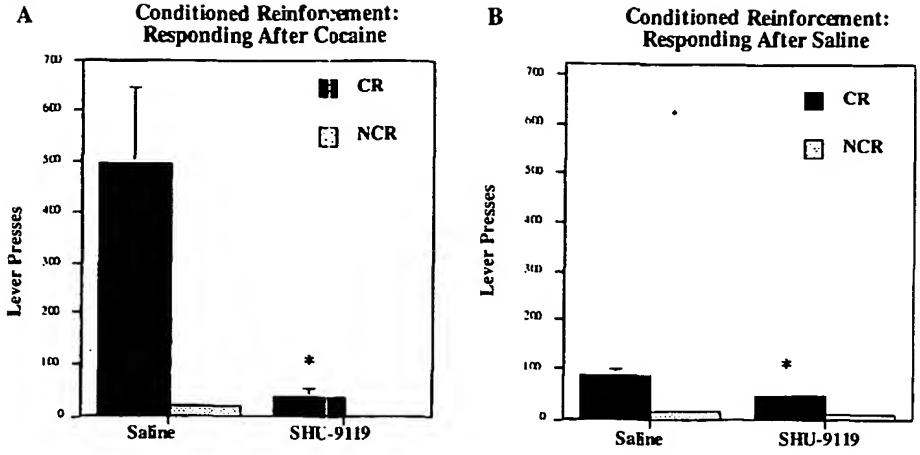


FIG. 3. Cocaine-enhanced conditioned reward is blocked by intra-accumbens infusion of SHU-9119. Rats were trained to associate a compound stimulus (tone + light) with water reward for 15 days. Responding for the conditioned reinforcer (CR lever) relative to the no CR stimulus (NCR lever) was measured after (A) cocaine (15 mg/kg) or (B) saline injections. Intra-accumbens SHU-9119 significantly blocked cocaine-enhanced responding for CR compared with the saline infusions. After saline, animals receiving intra-accumbens infusions of SHU-9119 (1 μg/0.5 μL) also made significantly fewer CR responses than subjects given intra-nucleus accumbens saline infusions. Bars represent the mean number of lever responses ± SEM, $n = 6,7$ for (A) and $n = 14$ for (B). *$P < 0.01$ SHU-9119 compared with saline-infused controls (ANOVA and Fisher's *post-hoc* test).

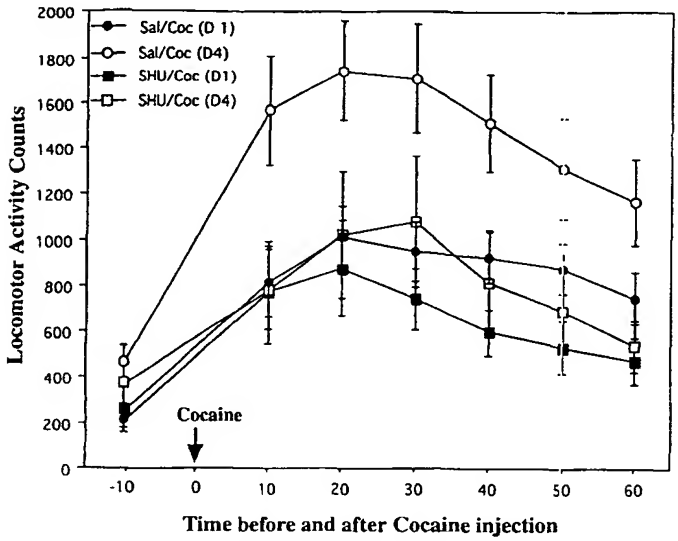


FIG. 4. Cocaine-induced locomotor activation is blocked by intra-accumbens infusion of SHU-9119. Rats received bilateral intra-nucleus accumbens infusions of SHU-9119 (1 μg/0.5 μL) or saline 30 min before administration of cocaine (15 mg/kg) for four consecutive days. Locomotor activity is shown 10 min prior to cocaine and for 10-min intervals after cocaine administration as indicated. The locomotor activity counts on day 1 (D1) and day 4 (D4) of cocaine administration are shown. The results are presented as mean activity counts ± SEM, $n = 13$ for saline + cocaine, and $n = 11$ for SHU-9119 + cocaine. *Post-hoc* comparisons revealed significant reductions in the SHU-9119 animals compared with saline animals at 10, 20, 40, 50 and 60 min ($P < 0.05$), and a trend at the 30 min time point after cocaine on day 4. No differences were observed between the groups on day 1 or before the cocaine infusions on day 1 or 4. $P < 0.05$ compared with vehicle-infused control (ANOVA and Fisher's *post-hoc* test).

accumbens SHU-9119 infusions selectively attenuated the ability of the stimulus to act as a CR.

The influence of SHU-9119 on the psychostimulant or locomotor-activating effects of cocaine was also determined. Figure 4 demonstrates the locomotor activity counts 10 min before and for the 60-min period after cocaine administration. Animals received intra-accumbens saline or SHU-9119 infusions 30 min prior to cocaine administration. The responses on day 1 and 4 of cocaine exposure are shown. There were significant differences between the groups that were observed over the 60-min post-cocaine injection period. Administration of cocaine for 4 days resulted in a significant increase in levels of locomotor activity relative to cocaine for 1 day demonstrating the locomotor-activating effects of repeated cocaine. Intra-accumbens infusion of SHU-9119 completely blocked the effects of repeated cocaine administration. SHU-9119 infusion did not influence baseline locomotor activity during the 10-min interval prior to cocaine administration (Fig. 4). In contrast to this antagonist effect, infusion of a melanocortin agonist, α-melanocyte-stimulating hormone (α-MSH), into nucleus accumbens (30 min before each cocaine treatment) significantly increased cocaine responsiveness [activity counts on day 5: vehicle = 1243 ± 172, α-MSH = 2204 ± 243, mean ± SEM ($n = 6$), $P < 0.05$ (Student's *t*-test)].

Locomotor-activating effects of cocaine are blocked in Agouti and MC4-R mutant mice

A role for the melanocortin system was further examined in Agouti mice, and the role of MC4-R was directly tested in MC4-R null mutant mice. In Agouti mice, ectopic expression of the agouti peptide results in blockade of melanocortin receptors in tissues other than skin, where the neuropeptide is normally found (Lu *et al.*, 1994). Both of these mutant models have been used to demonstrate that blockade of melanocortins and MC4-R increases food consumption and results in obesity via regulation of hypothalamic melanocortin receptors (Fan *et al.*, 1997; Huszar *et al.*, 1997). In control mice repeated administration of a low dose of cocaine (10 mg/kg), which did not significantly increase locomotor activity after a single dose, increased the locomotor-activating effects of cocaine approximately threefold by day 3 of cocaine treatment (Fig. 5A) (Hiroi *et al.*, 1997). Locomotor activation induced by cocaine was attenuated in the Agouti mice relative to control mice (Fig. 5A). This effect was significant only at the earlier time points studied (days 2 and 3), possibly due to incomplete inhibition of the melanocortin system by agouti protein. There was no significant difference in the response to saline between the Agouti mice and controls (Fig. 5B). A similar but more robust effect was observed in the MC4-R null mutant mice (−/−) relative to

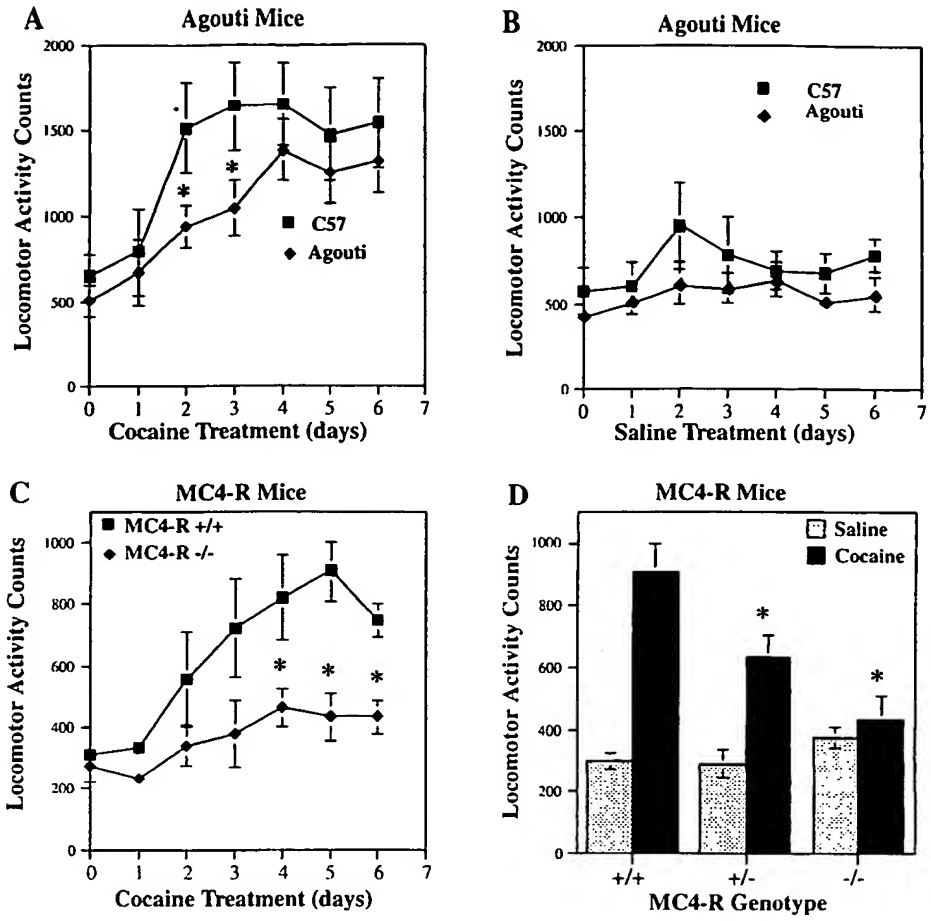


FIG. 5. Cocaine-induced locomotor activity is blocked in Agouti and MC4-R null mutant mice. (A, B) Agouti mice and C57BL/6 control mice were given cocaine (10 mg/kg) or saline on days 1–6, and levels of locomotor activity were measured. Day 0 reflects locomotor activity after 3 days of habituation to the chambers. Cocaine-induced locomotor activation was significantly reduced in the Agouti mice (A), but the response to saline administration (B) was not significantly different between Agouti and C57BL/6 mice. The results are presented as locomotor activity counts and are the mean ± SEM. The numbers of animals per group were: C57BL/6 + cocaine = 8; Agouti + cocaine = 9; C57BL/6 + saline = 6; Agouti + saline = 5. (C, D) MC4-R wild-type (+/+), heterozygous (+/−) and homozygous null mutant (−/−) littermates were also tested for cocaine-induced locomotor behavior. The response to cocaine on each day was significantly decreased in MC4-R −/− mice (C). (D) The response to either cocaine or saline treatment on day 5 only, demonstrating a significant, but intermediate, reduction in the MC4-R +/− mice, as well as complete inhibition of the cocaine response in the MC4-R −/− mice. The locomotor activity counts in response to cocaine in homozygous null mutant mice demonstrated no significant difference from locomotor activity counts in wild-type littermates receiving saline in all the days tested (data not shown). In addition, (D) demonstrates that there is no significant difference in baseline locomotor activity, in the absence of cocaine, between the MC4-R +/+, +/− and −/− littermates. The results shown are the first 10 min after cocaine administration and are presented as the mean locomotor activity counts ± SEM. The numbers of animals per group for cocaine treatment were: +/+ = 5; +/− = 7; and −/− = 10; and for saline treatment: +/+ = 4; +/− = 7; and −/− = 9. *$P < 0.05$ compared with the corresponding control (ANOVA and Fisher's *post-hoc* test).

their wild-type littermates (+/+). In the homozygous MC4-R −/− mice, cocaine-induced locomotor activation was completely abolished relative to +/+ mice (Fig. 5C), and an intermediate reduction in the cocaine response was observed in MC4-R +/− mice (Fig. 5D). In contrast, locomotor activity in response to saline administration was indistinguishable among wild-type littermates and MC4-R +/− and −/− mice (Fig. 4D).

MC4-R expression is upregulated by cocaine

The molecular and cellular interactions between the melanocortin and mesolimbic dopamine systems were examined to elucidate the mechanisms underlying the effects of melanocortin blockade on cocaine reward. Because MC4-R is one of the major melanocortin receptors expressed in the striatum (Alvaro *et al.*, 1996, 2003), and based on the results obtained in the MC4-R null mutant mice, efforts were focused on this receptor subtype. We first examined the influence of cocaine administration on expression of MC4-R mRNA by RNase protection analysis of dissected brain regions containing both the dorsal and ventral (nucleus accumbens) striatum. The entire striatum was used because more selective dissections of the nucleus accumbens alone did not provide sufficient amounts of mRNA for the RNase protection analysis. The results demonstrate that chronic cocaine administration results in a two- to threefold induction of MC4-R mRNA in striatum and smaller increases in the hypothalamus and hippocampus (Fig. 6A and B). There was no significant effect of cocaine treatment on expression of MC4-R mRNA in the septum or cerebral cortex (Fig. 6A and B). We have also found that this effect is dependent on chronic cocaine administration (Alvaro *et al.*, 2003).

The results of our behavioral studies indicate that the melanocortin–MC4-R system in nucleus accumbens contributes to both the primary and conditioned rewarding, as well as the locomotor-activating effects of cocaine, and that blockade of this neuropeptide system inhibits these behavioral responses to cocaine. To examine the cellular basis

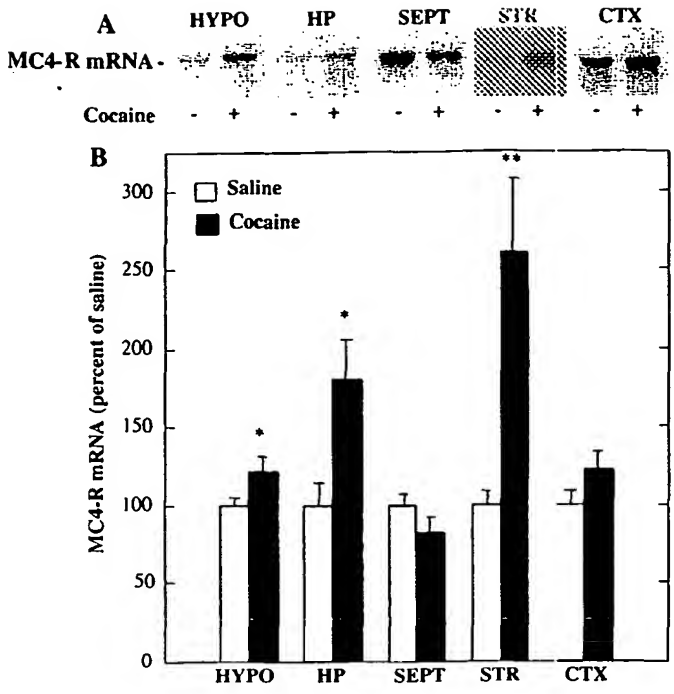


FIG. 6. Regulation of MC4-R mRNA by administration of cocaine. (A, B) Rats were given cocaine (15 mg/kg, twice daily for 14 days) and MC4-R mRNA was determined by RNase protection analysis. MC4-R mRNA was measured in hypothalamus (HYPO), hippocampus (HP), septum (SEPT), striatum (STR), and cerebral cortex (CTX). (A) Representative autoradiograms for each of the brain regions examined. (B) MC4-R mRNA was quantified by densitometry and the results are expressed as percent of control (saline). Each bar represents the mean ± SEM, $n = 6$. $^{*}P < 0.05$ or $^{**}P < 0.005$, compared with control (Student's *t*-test).

for the actions of the melanocortin system on cocaine-mediated behaviors, co-localization of MC4-R in prodynorphin- as well as in proenkephalin-containing neurons in the nucleus accumbens was examined. *In situ* hybridization analysis demonstrates the enrichment of MC4-R mRNA in dorsal striatum and nucleus accumbens (Fig. 7A). Double *in situ* hybridization analysis demonstrates that MC4-R is primarily co-localized with prodynorphin (Fig 7B), and is expressed with proenkephalin at a much lower rate. Quantitative analysis revealed that in the nucleus accumbens 79.1 ± 1.2% of cells positive for MC4-R mRNA also stained positive for prodynorphin (100 cells counted per rat, $n = 6$ rats, for a total of 600 cells), while only 15.3 ± 0.4% of cells positive for MC4-R mRNA also stained positive for proenkephalin (100 cells counted/rat, $n = 6$, for a total of 600 cells).

Discussion

Previous studies have demonstrated anatomical and functional interactions between the melanocortin- and dopamine-regulated signaling systems. The results of the present study provide the first direct evidence that the rewarding and locomotor-activating effects of cocaine are critically dependent on the activity of the melanocortin system in the nucleus accumbens. The requirement for melanocortins was demonstrated in three different behavioral models that are sensitive to the effects of cocaine on reinforcement processes, as well as analysis of the locomotor-activating effects of cocaine. Self-administration is regarded to be one of the most relevant behavioral models for assessing the reinforcing actions of cocaine (i.e. drug taking), and microinfusions of a melanocortin antagonist into the nucleus accumbens attenuated cocaine self-administration. Administration of SHU-9119 resulted in a downward shift in the number of lever presses in the dose–response to cocaine, as well as a rightward shift when expressed as total amount of cocaine self-administered. This suggests that SHU-9119 produces both a rate-decreasing effect as well as a competitive shift in responding that is overcome by higher doses of cocaine. Together, the results indicate that the primary reinforcing effects of cocaine are dependent on the melanocortin system in the nucleus accumbens. However, we cannot exclude the possibility that infusions of SHU-9119 also reached the dorsal striatum by traveling up the cannula track. Further studies must be conducted to test the effects of SHU-9119 in dorsal striatum and other brain regions.

The results of the current study are also consistent with a recent report demonstrating that infusion of a melanocortin agonist increases the acute reinforcing effects of amphetamine measured by self-stimulation of the lateral hypothalamus (Cabeza de Vaca *et al.*, 2002). However, there were several differences between the latter and present studies. First, for the hypothalamic self-stimulation study the melanocortin agonist infusions were made into the lateral ventricle, and it was not possible to determine the brain region that underlies the effects of the melanocortin system on psychostimulant responsiveness. Second, the effects of the melanocortin agonist on acute, but not

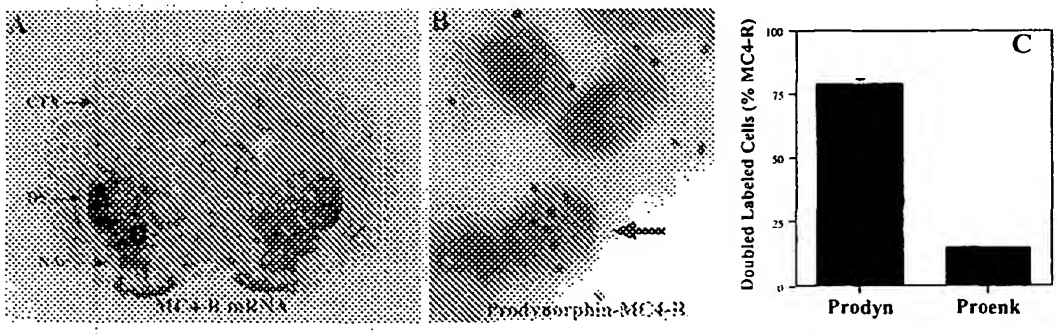


FIG. 7. Co-localization of MC4-R in prodynorphin-expressing neurons. (A) *In situ* hybridization analysis of MC4-R mRNA using a ^{35}S-labeled riboprobe demonstrates enrichment of MC4-R expression in the striatum, including the dorsal striatum (DS) and nucleus accumbens (NAc). Co-localization of ^{35}S-labeled MC4-R riboprobe and a digoxigenin-labeled prodynorphin riboprobe (B) in the nucleus accumbens was conducted according to standard procedures. Representative micrographs demonstrate ^{35}S-labeled MC4-R grains over (B) prodynorphin-expressing cells in the nucleus accumbens. (C) The number of MC4-R-positive cells that also express either prodynorphin or proenkepalin was determined. Co-localization of MC4-R with proenkephalin was also determined (not shown). A total of 600 MC4-R-positive cells was identified for each double-labeling condition (100 cells per rat, $n = 6$). The results are expressed as percent of MC4-R-positive cells that are also positive for either prodynorphin or proenkephalin.

repeated, amphetamine enhancement of self-stimulation was examined. This could also account for the lack of effect of SHU-9119 on acute amphetamine enhancement of self-stimulation of lateral hypothalamus (Cabeza de Vaca *et al.*, 2002) and is consistent with the lack of effect of SHU-9119 on the acute locomotor response to cocaine in the present study (i.e. day 1). All of the drug reward models of the current study used repeated administration of cocaine that results in sensitization of the behavioral actions of cocaine. Intra-accumbens infusions of SHU-9119 blocked cocaine-induced self-administration and locomotor sensitization. Taken together, the results of the current study demonstrate that upregulation of the nucleus accumbens melanocortin signaling is necessary for the rewarding effects of cocaine that are believed to contribute to aspects of drug addiction (Everitt & Wolf, 2002).

Similar results were obtained with the conditioned place preference and conditioned reinforcement paradigms that model the conditioned reinforcing and incentive motivational effects of drugs of abuse. Infusions of the melanocortin antagonist into the nucleus accumbens blocked cocaine-induced place preference and cocaine-enhanced responding for conditioned reinforcement, as well as the ability of the conditioned stimulus to act as a reinforcer. Inhibition of cocaine-induced place conditioning was observed whether the antagonist was infused before each pairing of a compartment with cocaine administration or only infused on the test day in the absence of cocaine. Moreover, the place conditioning studies indicate that infusion of the melanocortin antagonist causes avoidance to cocaine, although SHU-9119 infusions do not produce avoidance when administered in the absence of cocaine. The selective avoidance produced by the melanocortin antagonist on the test day could be due to an attenuation of cocaine's positive conditioned effect, thereby unmasking possible conditioned aversive actions of the psychostimulant (Carlezon *et al.*, 1998). Together these findings indicate that the heightened incentive qualities of drugs and drug-associated stimuli (i.e. conditioned reinforcing properties) are dramatically attenuated by blockade of nucleus accumbens melanocortin signaling, in addition to blockade of the primary reinforcing (i.e. unconditioned) effects of cocaine demonstrated in the self-administration studies. Moreover, the results suggest that blockade of the melanocortin system may attenuate the conditioned reinforcing properties of drugs of abuse that contribute to certain aspects of addiction.

The results also demonstrate that the melanocortin system is necessary for the development of cocaine-induced locomotor activation. Infusions of the melanocortin antagonist into nucleus accumbens completely blocked locomotor sensitization to cocaine. In contrast, SHU-9119 did not influence locomotor activity prior to administration of cocaine. Although the latter result suggests that SHU-9119 does not produce generalized sedation, we cannot rule out the possibility that rate-decreasing or sedation contribute to the effects of SHU-9119 in the operant or conditioned reinforcement paradigms. Similar effects were also observed in Agouti mice, in which ectopic expression of agouti protein blocks melanocortin receptors in brain and other tissues (Lu *et al.*, 1994). Locomotor activation in response to repeated cocaine administration was significantly decreased in the Agouti mice relative to controls, but there was no significant difference in baseline locomotor activity between Agouti and control mice after administration of saline. This demonstrates, by a complementary approach, that inhibition of the melanocortin system blocks behavioral responses to cocaine. The melanocortin antagonist, SHU-9119, used for the behavioral studies has affinity for both MC4-R as well as MC3-R (Hruby *et al.*, 1995), the other melanocortin receptor subtype expressed in the brain, although MC4-R is the predominant subtype expressed in the striatum and nucleus accumbens (Alvaro *et al.*, 1996). The role of the MC4-R subtype was directly demonstrated by studies showing that the locomotor-activating effects of cocaine were completely blocked in MC4-R homozygous null mutant mice and partially blocked in heterozygous null mutants. The effect of MC4-R mutation was specific to the cocaine response because there was no difference in locomotor activity following saline injections. These results provide strong evidence that MC4-R is the receptor subtype that mediates the actions of the melanocortin system on cocaine-induced locomotor sensitization. Future studies will examine the influence of the melanocortin system on responding to saline or cocaine challenge to examine the expression of locomotor sensitization in the absence of the melanocortin antagonist.

The molecular and cellular mechanisms underlying the actions of the melanocortin system in the responses to cocaine were also examined. We found that repeated administration of cocaine increases the expression of MC4-R mRNA, determined by RNase protection analysis. In a recent study we have found that administration of a low dose of morphine also upregulates the expression of MC4-R mRNA in striatum (Alvaro *et al.*, 2003). The observed upregulation of MC4-R in the striatum would be expected to enhance the behavioral effects of cocaine and could therefore be one mechanism contributing to the development of drug-induced behavioral sensitization. Previous studies have demonstrated that agonist-activation of the melanocortin system increases dopaminergic activity (Ryan & Isaacson, 1983; Torre *et al.*, 1986; Spruijt *et al.*, 1992; Lezcano *et al.*, 1995; Lindblom *et al.*, 2001; Florijn *et al.*, 1993a,b). Based on these reports, upregulation of MC4-R in the nucleus accumbens by cocaine treatment would be expected to increase dopaminergic-regulated neurotransmission and intracellular signaling, resulting in increased sensitivity to cocaine, which could be one mechanism contributing to drug-induced sensitization. Upregulation of MC4-R by repeated cocaine also provides indirect support for the hypothesis that this receptor subtype mediates the actions of the melanocortin antagonist on cocaine-induced behaviors. Because of the requirement for relatively large amounts of tissue and RNA for the RNase protection assay, expression of MC4-R mRNA was determined in the entire striatum. Additional studies will be required to examine the regulation of MC4-R mRNA in the nucleus accumbens to further test this hypothesis.

Previous reports of positive interactions between melanocortins and dopaminergic neurotransmission suggest a possible mechanism by which the melanocortin–MC4-R system could be involved in the actions of cocaine (Ryan & Isaacson, 1983; Torre *et al.*, 1986; Spruijt *et al.*, 1992; Florijn *et al.*, 1993a; Lezcano *et al.*, 1995). MC4-R, like the dopamine-D1 receptor, is positively coupled to the cAMP second messenger cascade (Gantz *et al.*, 1993; Mountjoy *et al.*, 1994). In addition, D1 receptor antagonist administration blocks cocaine-induced place preference and responding for conditioned reinforcement (Wolterink *et al.*, 1993; Shippenberg & Heidbreder, 1995). These findings suggest that activation of the MC4-R-coupled cAMP cascade could enhance D1 receptor function. In support of this hypothesis, the results of the double *in situ* hybridization studies demonstrate that MC4-R is co-localized with prodynorphin mRNA in nucleus accumbens medium spiny neurons. Given the high degree of D1 receptor localization with prodynorphin, this suggests that MC4-R is expressed in the same population of neurons that express the D1 receptor in nucleus accumbens. Thus, the shared post-receptor signaling pathways for MC4-R and D1 receptors, activation of the cAMP cascade, could account for their positive functional interactions. An alternative explanation is provided by studies demonstrating that α-MSH acts as an allosteric inhibitor of D1 receptors (Lezcano *et al.*, 1995), and upregulation of MC4-R and subsequent binding of

α-MSH could reduce the availability of this neuropeptide for this allosteric site.

The molecular and cellular mechanisms underlying cocaine-induced neural plasticity in the nucleus accumbens are reported to be critically dependent on adaptations of the dopamine and glutamate neurotransmitter systems (Hyman & Malenka, 2001; Nestler, 2001; Everitt & Wolf, 2002), and the results of the present study demonstrate a novel and important role for adaptation of the melanocortin neuropeptide system. The results demonstrating that the melanocortin antagonist blocks not only the primary reinforcing, but also the conditioned reinforcing, effects of cocaine raise the possibility that the melanocortin system may be involved in the ability of reward-related stimuli to induce compulsive drug-seeking behavior. Moreover, the results of this study support the possibility that an MC4-R antagonist could be used for the treatment of cocaine addiction by blocking the adaptive processes associated with addiction (Hyman & Malenka, 2001; Nestler, 2001; Everitt & Wolf, 2002). Future studies will examine the influence of small molecule antagonists that can be administered peripherally to block central melanocortin receptors to directly test this hypothesis. The discovery that melanocortins and MC4-R regulate eating behavior (Fan *et al.*, 1997; Huszar *et al.*, 1997) has stimulated the development of such drugs. It is also interesting to speculate that melanocortin regulation of feeding may be mediated, in part, via similar effects on brain reward systems that include the nucleus accumbens (Kelley & Berridge, 2002), in addition to actions on hypothalamic nuclei known to control eating homeostasis (Cowley *et al.*, 1999). Thus, drugs acting at MC4-R could prove useful for a wide array of disorders related to appetitive-motivated behavior.

Acknowledgements

Supported by grants (to R.S.D., E.J.N. and J.R.T.) from the National Institute on Drug Abuse, and a grant (to V.J.H.) from the US Public Health Service. We would also like to acknowledge Millenium Pharmaceuticals (Cambridge, MA, USA) for providing the MC4-R null mutant mice and Valyphone Phantharangsy for excellent technical support.

Abbreviations

CR, conditioned reinforcement; FR-1, fixed-ratio 1; MC4-R, melanocortin-4 receptor; MSH, melanocyte-stimulating hormone; NCR, non-conditioned reinforcement; POMC, proopiomelanocortin.

References

Adan, R. & Gispen, W.H. (1997) Brain melanocortin receptors: from cloning to function. *Peptides*, **18**, 1279–1287.

Alvaro, J.D., Hsu, R. & Duman, R.S. (2003) Chronic cocaine administration increases the expression of MC4-R in rat neostriatum. *J. Pharmacol. Exp. Ther.*, **304**, 391–399.

Alvaro, J., Tatro, J.B. & Duman, R.S. (1997) Melanocortins and opiate addiction. *Life Sci.*, **61**, 1–9.

Alvaro, J., Tatro, J.B., Quillan, J.M., Fogliano, M., Eisenhart, M., Lerner, M.R., Nestler, E.J. & Duman, R.S. (1996) Morphine down-regulates melanocortin-4 receptor expression in brain regions that mediate opiate addiction. *Mol. Pharmacol.*, **50**, 583–591.

Cabeza de Vaca, S., Kim, G.-Y. & Carr, K.D. (2002) The melanocortin receptor agonist MTII augments the rewarding effect of amphetamine ad-libitum-fed and food-restricted rats. *Psychopharmacol.*, **161**, 77–85.

Carlezon, W.J., Thome, J., Olson, V.G., Lane-Ladd, S.B., Brodkin, E.S., Hiroi, N., Duman, R.S., Neve, R.L. & Nestler, E.J. (1998) Regulation of cocaine reward by CREB. *Science*, **282**, 2272–2275.

Contreras, P. & Takemori, A.E. (1984) Antagonism of morphine-induced analgesia tolerance and dependence by α-melanocyte-stimulation hormone. *JPET*, **229**, 21–26.

Cowley, M., Pronchuk, N., Fan, W., Dinulescu, D.M., Colmers, W.F. & Cone, R.D. (1999) Integration of NPY, AGRP, and melanocortin signals in the hypothalalmic paraventricular nucleus: evidence of a cellular basis for the adipostat. *Neuron*, **24**, 155–163.

Cremer, M., Sanchez, M.S. & Celis, M.E. (2000) Structure-activity studies of α-melanotropin fragments on cAMP production in striatal slices. *Peptides*, **21**, 803–806.

Eskay, R., Giraud, P., Oliver, C. & Brown-Stein, M.J. (1979) Distribution of α-melanocyte-stimulating hormone in the rat brain: evidence that α-MSH-containing cells in the arcuate region send projections to extrahypothalamic areas. *Brain Res.*, **178**, 55–67.

Everitt, B. & Wolf, M.E. (2002) Psychomotor stimulant addiction: a neural systems perspective. *J. Neurosci.*, **22**, 3312–3320.

Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J. & Cone, R.D. (1997) Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature*, **385**, 165–168.

Florijn, W., Holtmaat, A.J., de Lang, H., Spierenburg, H., Gispen, W.H. & Versteeg, D.H. (1993a) Peptide-induced grooming behavior and caudate nucleus dopamine release. *Brain Res.*, **625**, 169–172.

Florijn, W., Mulder, A.H., Versteeg, D.H. & Gispen, W.H. (1993b) Adrenocorticotropin/alpha-melanocyte-stimulation hormone (ACTH/MSH)-like peptides modulate adenylate cyclase activity in rat brain slices: evidence for an ACTH/MSH receptor-coupled mechanism. *J. Neurochem.*, **60**, 2204–2211.

Gantz, I., Konda, Y., Tashiro, T., Shimoto, H., Miwa, H., Munsert, G., De Watson, S.J.I., Valle, J. & Yamada, T. (1993) Molecular cloning of a novel melanocortin receptor. *J. Biol. Chem.*, **268**, 8246–8250.

Hiroi, N., Brown, J.R., Haile, C.N., Ye, H., Greenberg, M.E. & Nestler, E.J. (1997) FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. *PNAS*, **94**, 10397–10402.

Horger, B., Iyasere, C.A., Berhow, M.T., Messer, C.J., Nestler, E.J. & Taylor, J.R. (1999) Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. *J. Neurosci.*, **19**, 4110–4122.

Hruby, V., Lu, D., Sharma, S.D., Castrucci, A.L., Kesterson, R.A., al-Obeidi, F.A., Hadley, M.E. & Cone, R.D. (1995) Cyclic lactam alpha-melanotropin analogues of Ac-Nle4-cyclo [Asp5, D-Phe7, Lys10] alpha-melanocyte-stimulating hormone-(4–10)-NH2 with bulky aromatic amino acids at position 7 show high antagonist potency and selectivity at specific melanocortin receptors. *J. Med. Chem.*, **38**, 3454–3461.

Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu, W., Kesterson, R.A., Boston, B.A., Cone, R.D., Smith, F.J., Campfield, L.A., Burn, P. & Lee, F. (1997) Targeted disruption of themelanocortin-4 receptor results in obesity in mice. *Cell*, **88**, 131–141.

Hyman, S. & Malenka, R.C. (2001) Addiction and the brain: the neurobiology of compulsion and its persistence. *Nat. Rev. Neuro.*, **2**, 695–703.

Jacobowitz, D. & O'Donohue, T.L. (1978) g-Melanocyte stimulating hormone: immunohistochemical identification and mapping in neurons in brain. *PNAS*, **75**, 6300–6304.

Jentsch, J.P. & Taylor, J.R. (1999) Impulsivity resulting from frontostriatal dysfunction in drug abuse: implications for the control over behavior by reward-related stimuli. *Psychopharmacol.*, **146**, 373–390.

Kelley, A.E. & Berridge, K.C. (2002) The neuroscience of natural rewards: relevance to addictive drugs. *J. Neurosci.*, **22**, 3306–3311.

Lezcano, N., DeBarioglio, S.R. & Celis, M.E. (1995) α-MSH changes cyclic AMP levels in rat brain slices by an interaction with the D1 dopamine receptor. *Peptides*, **16**, 133–137.

Lezcano, N., Salvatierra, N.A. & Celis, M.E. (1995) α-Melanotropin hormone inhibits the binding of [3H]SCH 23390 to the dopamine D1 receptor in vitro. *Eur. J. Pharmacol.*, **363**, 211–215.

Lindblom, J., Opmane, B., Mutulis, F., Mutule, I., Petrovska, R., Klusa, V., Bergstrom, L. & Wikberg, J.E. (2001) The MC4 receptor mediates alpha-MSH induced release of nucleus accumbens dopamine. *Neuroreport*, **12**, 2155–2158.

Lu, D., Willard, D., Patel, I.R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R.P., Wilkison, W.O & Cone, R.D. (1994) Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature*, **371**, 799–802.

Mountjoy, K., Mortrud, M.T., Low, M.J., Simerly, R.B. & Cone, R.D. (1994) Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol. Endocrinol.*, **8**, 1298–1308.

Nestler, E. (2001) Total recall – the memory of addiction. *Science*, **292**, 2266–2267.

Piazza, P., Deroche-Gamonent, V., Rouge-Pont, F. & LeMoal, M. (2000) Vertical shifts in self-administration dose–response functions predict a drug-vulnerable phenotype predisposed to addiction. *J. Neurosci.*, **11**, 4226–4232.

Robinson, T.E. & Berridge, K.C. (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res. Brain Res. Rev.*, **18**, 247–291.

Ryan, J. & Isaacson, R.L. (1983) Intra-accumbens injections of ACTH induce excessive grooming in rats. *Physiol. Psychol.*, **11**, 54–58.

Samyai, Z., Vecsernyes, M., Julesz, J., Szabo, G. & Telegdy, G. (1992) Effects of cocaine and pimozide on plasma and brain alpha-melanocyte-stimulating hormone levels in rats. *Neuroendocrinology*, **55**, 9–13.

Shippenberg, T. & Heidbreder, C. (1995) Sensitization to the conditioned rewarding effects of cocaine: pharmacological and temporal characteristics. *JPET*, **273**, 808–815.

Spruijt, B., VanHooff, J.A. & Gispen, W.H. (1992) Ethology and neurobiology of grooming behavior. *Physiol. Rev.*, **72**, 825–852.

Stone, D., Walsh, J. & Benes, F.M. (1999) Localization of cells preferentially expressing GAD (67) with negligible GAD (65) transcripts in the rat hippocampus. A double in situ hybridization study. *Mol. Brain Res.*, **71**, 201–209.

Szekaly, J., Miglecz, E., Dunai-Kovacs, Z., Tarnawa, I., Ronai, A.Z., Graf, L. & Bajusz, S. (1979) Attenuation of morphine tolerance and dependence by a-melanocyte stimulating hormone (α-MSH). *Life Sci.*, **24**, 1931–1938.

Taylor, J.R. & Horger, B.A. (1999) Enhanced responding for conditioned reward produced by intra-accumbens amphetamine is potentiated after cocaine sensitization. *Psychopharmacol.*, **142**, 31–40.

Taylor, J.R. & Robbins, T.W. (1984) Enhanced behavioural control by conditioned reinforcers following microinjections of d-amphetamine into the nucleus accumbens. *Psychopharmacol.*, **84**, 405–412.

Taylor, J.R. & Robbins, T.W. (1986) 6-Hydroxydopamine lesions of the nucleus accumbens, but not of the caudate nucleus, attenuate enhanced responding with reward-related stimuli produced by intra-accumbens d-amphetamine. *Psychopharmacol.*, **90**, 390–397.

Tong, Y. & Pelletier, G. (1992) Role of dopamine in the regulation of pro-opiomelanocortin (POMC) mRNA levels in the arcuate nucleus and pituitary gland of the female rat as studied by in situ hybridization. *Mol. Brain Res.*, **15**, 27–32.

Torre, E. & Celis, M.E. (1986) Alpha-MSH injected into the substantia nigra or intraventricularly alters behavior and the striatal dopaminergic activity. *Neurochem. Int.*, **9**, 85–89.

Van der Kooy, D. (1987) *Place Conditioning: a Simple and Effective Method for Assessing the Motivational Properties of Abused Drugs*. Springer, New York.

Wolterink, G., Phillips, G., Cador, M., Donselaar-Wolterink, I., Robbins, T.W. & Everitt, B.J. (1993) Relative roles of ventral striatal D1 and D2 dopamine receptors in responding with conditioned reinforcement. *Psychopharmacol.*, **110**, 355–364.

Targeted Disruption of the Melanocortin-4 Receptor Results in Obesity in Mice

Dennis Huszar,* Catherine A. Lynch,*
Victoria Fairchild-Huntress,*
Judy H. Dunmore,* Qing Fang,*
Lucy R. Berkemeier,* Wei Gu,*
Robert A. Kesterson,† Bruce A. Boston,‡
Roger D. Cone,† Francoise J. Smith,§
L. Arthur Campfield,§ Paul Burn,§
and Frank Lee*
*Millennium Pharmaceuticals, Inc.
640 Memorial Drive
Cambridge, Massachusetts 02139
†Vollum Institute for Advanced Biomedical Research
‡Department of Pediatrics
Oregon Health Sciences University
Portland, Oregon 97201
§Hoffmann–La Roche, Inc.
Department of Metabolic Diseases
Nutley, New Jersey 07110

Summary

The melanocortin-4 receptor (MC4-R) is a G protein–coupled, seven-transmembrane receptor expressed in the brain. Inactivation of this receptor by gene targeting results in mice that develop a maturity onset obesity syndrome associated with hyperphagia, hyperinsulinemia, and hyperglycemia. This syndrome recapitulates several of the characteristic features of the *agouti* obesity syndrome, which results from ectopic expression of agouti protein, a pigmentation factor normally expressed in the skin. Our data identify a novel signaling pathway in the mouse for body weight regulation and support a model in which the primary mechanism by which agouti induces obesity is chronic antagonism of the MC4-R.

Introduction

Over the past few years, there has been considerable progress in identification and characterization of the mutations underlying five monogenic murine models of obesity: obese (*ob*), diabetes (*db*), fat (*fat*), tubby (*tub*), and obese yellow (e.g., A^y). In particular, cloning of the *ob* and *db* genes has led to identification of a signaling system that monitors the status of energy stores in the body. The *ob* gene product, leptin, is a circulating protein secreted by adipose tissue that communicates body fat content to the brain through the OB receptor (OB-R), the product of the *db* gene. The OB-R is a member of the class 1 cytokine receptor family expressed in the hypothalamus and other tissues (Zhang et al., 1994; Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Tartaglia et al., 1995; Baumann et al., 1996; Chen et al., 1996; Chua et al., 1996; Ghilardi et al., 1996; Lee et al., 1996).

The identification of the leptin signaling pathway represents a significant advance in the elucidation of the mechanisms by which body weight and energy expenditure are regulated. However, it is clear that the control of body weight is a complex process that will likely involve the interplay of additional, as yet undefined, neural circuits and molecular mechanisms (Friedman and Leibel, 1992; Campfield et al., 1996; Spiegelman and Flier, 1996). The existence of one such pathway, involving signaling via neural melanocortin receptors, has been hypothesized on the basis of studies of the action of the agouti pigmentation factor (Lu et al., 1994).

Agouti, a 131 amino acid secreted protein expressed exclusively within the hair follicle, regulates the synthesis of brown-black (eumelanin) and yellow-red (phaeomelanin) pigment to produce the wild-type (agouti) pattern of coat coloration: black hairs with a subapical yellow band (Bultman et al., 1992; Miller et al., 1993; reviewed in Silvers, 1979). Agouti acts in a paracrine fashion on melanocytes (Silvers and Russell, 1955) to induce a switch from eumelanin to phaeomelanin synthesis by antagonism of the melanocortin-1 receptor (MC1-R) (Lu et al., 1994), also known as the melanocyte-stimulating hormone receptor (MSH-R), a G protein–coupled, seven-transmembrane receptor (Mountjoy et al., 1992; Chhajlani and Wikberg, 1992; Robbins et al., 1993).

Dominant alleles of the *agouti* locus resulting in widespread ectopic expression of agouti give rise to a pleiotropic obesity syndrome referred to as the obese yellow, or *agouti*, syndrome (Bultman et al., 1992; Michaud et al., 1993; Miller et al., 1993; Duhl et al., 1994; Michaud et al., 1994a; 1994b). The best characterized and most dominant of the allele series are the lethal yellow (A^y) and viable yellow (A^{vy}) mutants. Obese yellow mice are characterized by maturity-onset obesity (in contrast to the early-onset obesity of *ob* and *db* mice), hyperinsulinemia, hyperglycemia in males, and yellow coat color (reviewed by Yen et al., 1994). The mice are hyperphagic (Frigeri et al., 1988; Shimizu et al., 1989) and display increased rates of hepatic lipogenesis (Yen et al., 1976b) and decreased rates of lipolysis in adipocytes (Yen et al., 1970). The increased adiposity of dominant agouti mutants is primarily due to fat cell hypertrophy, as is true of most human forms of obesity, rather than the more prevalent hyperplasia characteristic of *ob* and *db* mice (Johnson and Hirsch, 1972). In addition, obese yellow mice are unique among rodent obesity models in exhibiting a slight increase in linear growth, in contrast to *ob* and *db* mice, which are somewhat shorter than their wild-type littermates (Heston and Vlahakis, 1962; Wolff, 1963).

The dominant *agouti* alleles that give rise to obesity result in constitutive deregulated synthesis of wild-type agouti protein within the hair follicle, accounting for the yellow coat color, and ectopic expression of agouti throughout the animal, presumably accounting for the other characteristics of the pleiotropic obesity syndrome (Bultman et al., 1992; Michaud et al., 1993; Miller et al., 1993; Duhl et al., 1994). That this phenotype is directly related to ectopic expression of agouti, rather than to other potential effects of the various agouti promoter mutations, is supported by the demonstration

that transgenic mice ectopically expressing agouti from ubiquitous promoters recapitulate the obese hyperinsulinemic phenotype (Klebig et al., 1995; Perry et al., 1995). Furthermore, the obesity phenotype is not dependent on synthesis of yellow pigment, or disruption of MC1-R function, since A^{vy} mice carrying the gain of function MC1-R mutation somber (E^{so}) are black yet still obese (Wolff et al., 1978) and yellow *e/e* mice, which lack MC1-R function (Robbins et al., 1993), are not obese (Searle, 1968).

One hypothesis for the mechanism by which ectopic agouti expression induces obesity is aberrant antagonism of melanocortin receptors expressed in regions of the brain known to be involved in regulating feeding (Lu et al., 1994; Mountjoy et al., 1994). In addition to the MC1-R, four other members of the melanocortin receptor family have been identified: the MC2-R (Mountjoy et al., 1992), MC3-R (Gantz et al., 1993a; Roselli-Rehfuss et al., 1993), MC4-R (Gantz et al., 1993b; Mountjoy et al., 1994), and MC5-R (Chhajlani et al., 1993; Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994). All are G protein–coupled receptors that activate adenylyl cyclase and bind ligands collectively referred to as the melanocortins (e.g., adrenocorticotrophin [ACTH] and the α-, β-, and γ-melanocyte–stimulating hormones) derived from the precursor proopiomelanocortin (POMC). The MC2-R is the ACTH receptor, expressed primarily in the adrenal cortex (Mountjoy et al., 1992). The MC3-R and MC4-R are neural melanocortin receptors. The MC3-R is expressed in the brain in regions of the hypothalamus and limbic system, as well as in the placenta and gut (Gantz et al., 1993a; Roselli-Rehfuss et al., 1993), whereas expression of the MC4-R is restricted primarily to the brain, where it is widely expressed (Gantz et al., 1993b; Mountjoy et al., 1994; reviewed in Low et al., 1994). The MC5-R, on the other hand, is widely expressed at low levels throughout the body (Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994). To test for agouti antagonism, Lu et al. (1994) expressed melanocortin receptors in 293 cells and assayed the ability of agouti to inhibit α-MSH–induced stimulation of adenylyl cyclase activity. The results demonstrated that agouti was a high affinity antagonist of both the MC1 and MC4 receptors in vitro, but not of the MC3 or MC5 receptors, suggesting that agouti antagonism of the MC4-R could be the cause of the *agouti* obesity syndrome.

To evaluate whether signaling via the MC4-R plays a role in the regulation of body weight, mice lacking the receptor were generated by gene targeting in embryonic stem (ES) cells. Our results show that absence of the MC4-R produces an obesity syndrome strikingly reminiscent of the *agouti* syndrome, defining a novel function for the MC4-R in the regulation of energy balance and supporting aberrant antagonism of the MC4-R as the primary cause of the *agouti* obesity syndrome.

Results

Generation of MC4-R–Deficient Mice

The murine MC4-R gene consists of approximately 1 kb of coding sequence contained within a single exon

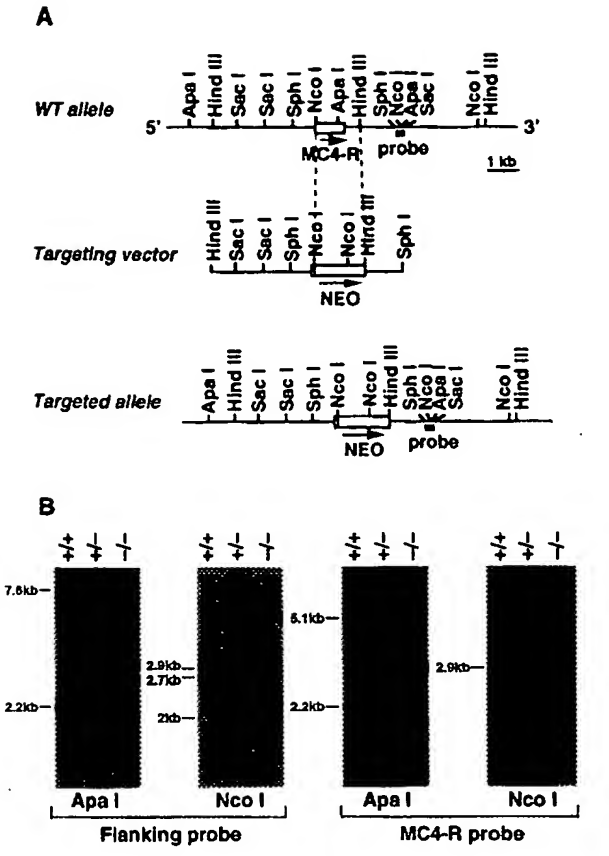


Figure 1. Deletion of the Mouse MC4-R

(A) Schematic diagrams and partial restriction maps of the MC4-R locus, the MC4-R targeting vector, and the predicted structure of the MC4-R locus following homologous recombination with the targeting vector. The open box represents MC4-R coding sequences, the closed box is the 200 bp SphI–SacI flanking probe used to identify the targeted MC4-R locus, and the shaded box indicates the PGK–*neo* expression cassette. The arrows indicate the direction of transcription.

(B) Autoradiogram of a Southern blot analysis of tail DNA from F2 progeny. Genomic DNA was digested with ApaI or NcoI, as indicated, and hybridized with the radiolabeled probe shown in (A), then stripped and rehybridized with a radiolabeled probe consisting of the human MC4-R coding sequence. +/+, +/−, and −/− denote DNA from wild-type, heterozygous, and homozygous F2 littermates, respectively.

(Figure 1A). A targeting vector was designed to delete virtually all MC4-R coding sequence following homologous recombination with the locus in embryonic stem cells. As shown in Figure 1A, the vector consists of a total of approximately 4.5 kb of strain 129/Sv mouse genomic DNA flanking a deletion of 1.5 kb. This deletion extends from the NcoI site located approximately 20 nucleotides downstream of the MC4-R initiation codon to the HindIII site situated approximately 0.5 kb 3′ of the gene. The deleted MC4-R sequences have been replaced by the *neo* gene under the control of the phosphoglycerate kinase-1 (PGK-1) promoter.

A total of 809 G418-resistant colonies were screened for homologous recombination by Southern blot hybridization of ApaI-digested genomic DNA with the flanking probe shown in Figure 1A. One clone showed the predicted 7.6 kb targeted ApaI DNA fragment in addition

to the expected 2.2 kb wild-type fragment. Injection of this clone into C57BL/6J blastocysts produced several male chimeras that, when bred to C57BL/6J females, transmitted the targeted MC4-R allele to their F1 129/B6 offspring. F1 heterozygotes were interbred and their offspring genotyped by Southern blot hybridization of ApaI- or NcoI-digested tail DNA with the flanking probe. As described above, ApaI digestion generates a wild-type fragment of 2.2 kb and a targeted fragment of 7.6 kb (note that this 7.6 kb is distinct from a background band of slightly lower molecular weight that is present in all samples; see Figure 1B). NcoI digestion generates two wild-type fragments of 2.7 and 2.9 kb, since the NcoI site is situated within the sequences recognized by the flanking probe (Figures 1A and 1B). The 2.7 kb NcoI fragment represents genomic sequences extending 3′ of the probe that are unaffected by MC4-R targeting, whereas the 2.9 kb band includes the MC4-R gene sequences. Following targeting, this latter fragment is reduced to a 2 kb band diagnostic of the mutated MC4-R allele. As shown in Figure 1B, heterozygous intercrosses produced homozygous mutant, heterozygous, and wild-type F2 progeny. To verify deletion of the MC4-R gene in homozygous mutants, the filters were stripped and rehybridized with an MC4-R probe. No MC4-R hybridization was detected in homozygous mutant mice, whereas the predicted 2.2 kb and 5.1 kb ApaI bands (ApaI cuts within the MC4-R gene generating two MC4-R–containing fragments; see Figure 1A) and 2.9 kb NcoI fragment were observed in both heterozygous and wild-type littermates (Figure 1B).

Body Weight and Size of MC4-R–Deficient Mice

F2 animals were maintained on a chow diet ad libitum and their weights monitored regularly. The weights of MC4-R–deficient mice and their wild-type littermates were largely indistinguishable for the first 4 weeks of life. However, by approximately 5 weeks of age, most of the homozygous mutants, both males and females, were heavier than their wild-type siblings of the same sex, and by 7 weeks of age all of the null mutants were heavier than the controls (Figures 2A and 2C). By 15 weeks of age, homozygous mutant females were on average twice as heavy as their wild-type siblings, while homozygous mutant males were approximately 50% heavier than wild-type controls. By about 24 weeks of age the weight of female null mice averaged approximately 63 grams (n = 3), and males averaged approximately 65 grams (n = 8). Mice heterozygous for MC4-R deletion showed a weight gain intermediate to that seen in wild-type and homozygous mutant sibs (Figures 2B and 2D), demonstrating a gene dosage effect of MC4-R ablation on body weight regulation.

To determine whether mice lacking the MC4-R exhibited alterations in linear growth, body length (naso–anal) measurements of F2 progeny were taken at approximately 19 weeks of age (between 132 and 138 days). As shown in Figure 3, MC4-R–deficient mice are significantly longer than wild-type controls. The mean length of homozygous mutant females is increased approximately 11% relative to wild-type F2 mice, and heterozygous females are approximately 7% longer than controls. Male homozygotes and heterozygotes are approximately 8% and 2.5% longer than controls, respectively.

Food Consumption

To determine whether food consumption was increased in mice lacking the MC4-R, homozygous mutant females and wild-type F2 controls were monitored for food intake over a two-week period. A^y mutants, on a C57BL/6J background, and C57BL/6J controls were also monitored. As previously documented (Frigeri et al., 1988; Shimizu et al., 1989), A^y mice were hyperphagic, eating 36% more than C57BL/6J controls. Absence of the MC4-R also resulted in a significant increase (46%) in food consumption over wild-type F2 controls (Figure 4).

Serum Glucose, Insulin, and Leptin Concentrations

Blood was collected from MC4-R–deficient mice and wild-type controls over three time intervals (4–8 weeks, 10–14 weeks, and 17–23 weeks) and serum assayed for glucose and insulin levels. Serum glucose levels were essentially unchanged in females heterozygous or homozygous for MC4-R deletion, but both heterozygous and homozygous males were hyperglycemic (Figures 5A and 5B). This was first evident for homozygous males at the 10–14 week interval, at which time glucose levels were elevated over 2-fold above controls, to 390 mg/dl, but heterozygous mutants showed only a slight elevation of serum glucose at this age. By 17–23 weeks of age, both heterozygous and homozygous male mutant mice showed a doubling of normal serum glucose levels (334 and 361 mg/dl, respectively) relative to controls (156 mg/dl).

Both male and female mutant mice were hyperinsulinemic (Figures 5C and 5D). Inceases in insulin levels by 9-fold and 5-fold were evident in the sera of homozygous mutant females and males, respectively, at 4–8 weeks of age. These levels increased dramatically over time, such that by 17–23 weeks of age the mean concentration of insulin in the serum of homozygous mutant females was approximately 65 ng/ml, and for males approximately 130 ng/ml, representing appoximately 60- and 14-fold increases, respectively, over insulin levels in F2 wild-type controls. Heterozygous mutants were also hyperinsulinemic, although less so than homozygous mutants. For both male and female heterozygotes, a significant difference in insulin levels relative to controls was first observed at the 10–14 week interval. By 17–23 weeks, mean insulin levels of heterozygotes were elevated to approximately 10 ng/ml (females) and 85 ng/ml (males).

In addition to glucose and insulin, serum leptin and corticosterone levels were also determined. Leptin levels are elevated in A^y mice (Maffei et al., 1995; Mizuno et al., 1996), indicating that the syndrome does not result from defects in leptin production. Consistent with the postulated role of leptin in communicating body fat depot contents to the brain (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Campfield et al., 1996), leptin is also elevated in MC4-R–deficient mice (Figures 5E and 5F). At 4–8 weeks of age, leptin was elevated 4.5-fold and 1.5-fold in the serum of female

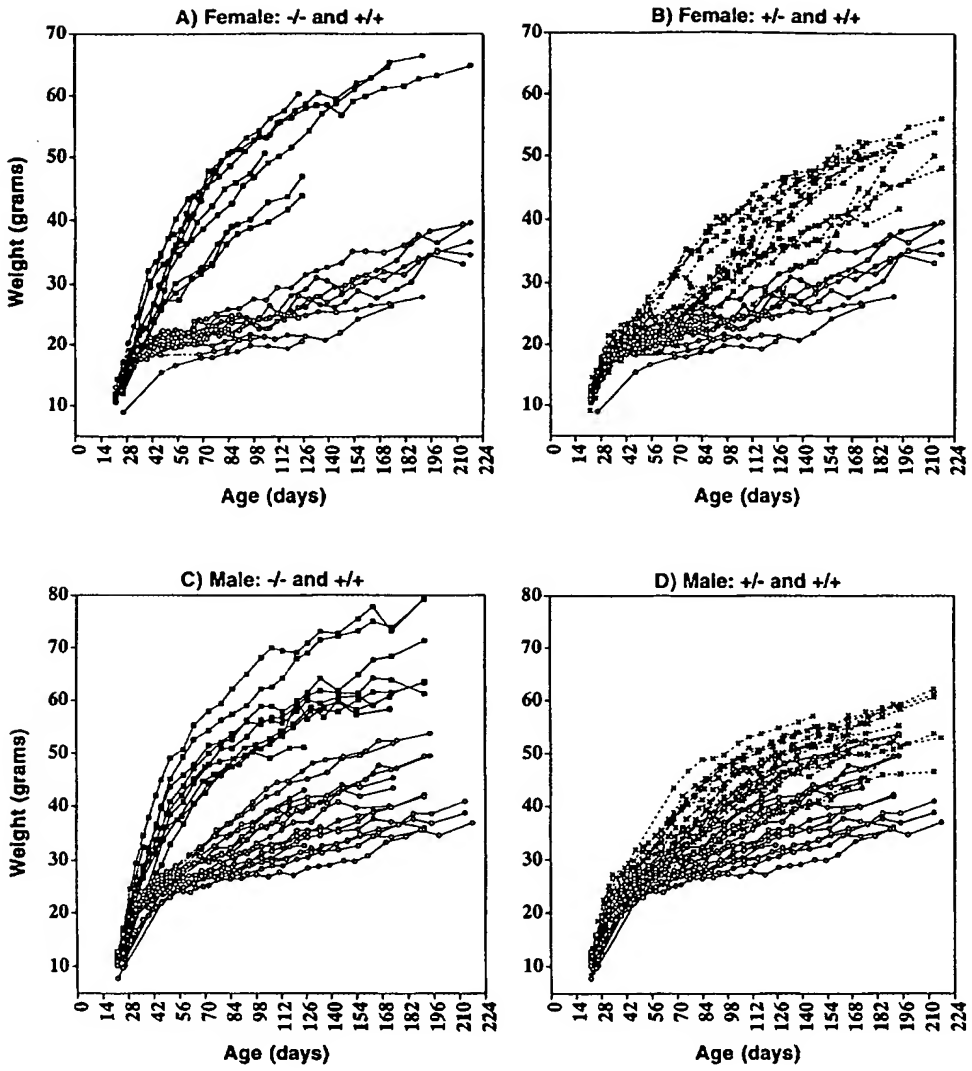


Figure 2. Weight Gain of MC4-R–Deficient Mice and Control Littermates

Each line represents the weight gain of an individual mouse.

(A) Weight gain of female homozygous (−/−) mutant mice (closed squares) and wild-type (+/+) F2 controls (open circles). The weights of 9 homozygous and 12 control mice were taken at the times indicated.

(B) Weight gain of female heterozygous (+/−) mutant mice (x) and wild-type (+/+) F2 controls (open circles). The weights of 18 heterozygous and 12 control mice were taken at the times indicated.

(C) Weight gain of male homozygous (−/−) mutant mice (closed squares) and wild-type (+/+) F2 controls (open circles). The weights of 9 homozygous and 17 control mice were taken at the times indicated.

(D) Weight gain of male heterozygous (+/−) mutant mice (x) and wild-type (+/+) F2 controls (open circles). The weights of 18 heterozygous and 17 control mice were taken at the times indicated.

and male homozygous mutants, respectively, relative to wild-type controls. By 17–23 weeks of age serum leptin levels in females had reached approximately 97 ng/ml, in males approximately 58 ng/ml, representing increases of 6.5- and 2.5-fold, respectively. Heterozygous mice, for the most part, showed leptin levels intermediate between that observed for wild-type mice and homozygous mutants.

Since glucocorticoids can profoundly affect weight homeostasis and somatic growth, basal serum corticosterone was measured in three sets of sex-matched littermates, each containing a wild-type, heterozygous, and homozygous mutant animal (Figure 6). No effect of MC4-R gene knockout on basal corticosterone levels was detected.

POMC Gene Expression

To assess whether the observed effects of MC4-R deletion on weight homeostasis could be attributed to the induction of compensatory changes in expression of the sole known source of ligand for the MC4-R, the POMC gene, central POMC gene expression was examined in wild-type mice, mice heterozygous for MC4-R deletion, and homozygous mutants by in situ hybridization (Figures 7D–7F). No new sites of POMC gene expression and no consistent change in the levels of POMC mRNA

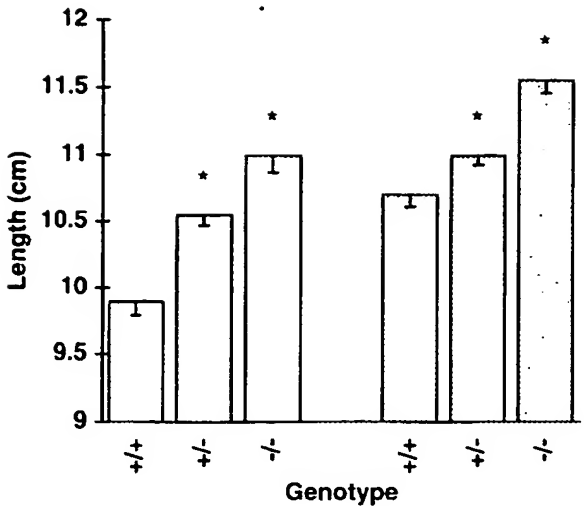


Figure 3. Increased Linear Growth of MC4-R–Deficient Mice

The body length of female (open bars) and male (hatched bars) was measured at approximately 19 weeks of age (between 132 and 138 days). The bars indicate the mean length of 12 wild-type (+/+), 14 heterozygous (+/−), and 9 homozygous mutant (−/−) female F2 mice, and 15 wild-type, 20 heterozygous, and 9 homozygous mutant male F2 mice. Error bars represent the standard error of the mean, and the asterisks denote significant difference ($p < 0.02$ by two-tailed Student t test) compared to the wild-type value within a similar sex.

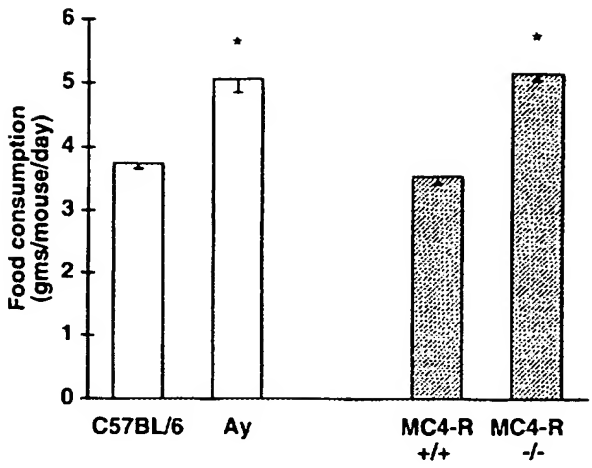


Figure 4. Mice Lacking the MC4-R Are Hyperphagic

The food intake of female mice housed in pairs was measured every weekday over a two-week period. The open bars represent the mean of eight measurements on one cage each of two A^y and two control C57BL/6 mice, at 9 weeks of age. The hatched bars represent the mean of eight measurements on each of two cages of two homozygous mutant mice (−/−) and two F2 wild-type controls (+/+). Two of each of the mutant mice and F2 controls were 15 weeks of age; the remaining two of both genotypes were each 17.5 and 20.5 weeks of age. Error bars represent the standard error of the mean, and the asterisks denote significant difference ($p < 0.01$ by two-tailed Student t test) of either A^y compared to C57BL/6 or MC4-R −/− homozygous mutants compared to MC4-R +/+ wild-type F2 mice.

in its primary site of expression, the arcuate nucleus of the hypothalamus, were detected by this assay. In addition, no gross neuroanatomical defects were observed in thionin-stained brain sections from heterozygous or homozygous mutant MC4-R–deficient animals by histological analysis (Figures 7A–7C).

Discussion

In this study, we have generated mice lacking expression of the MC4-R by gene targeting, and we report that a maturity onset obesity syndrome associated with hyperphagia, hyperinsulinemia, and hyperglycemia occurs in the absence of the receptor. This syndrome is quite distinct from that elicited by mutations of the *ob* and *db* loci, in which the inability to produce leptin (*ob/ob*) or respond to it (*db/db*) results in an early-onset obesity and extreme hyperphagia and hyperinsulinemia. In addition, in *ob* and *db* mice, basal- and stress-activated corticosterone levels are significantly elevated (Dubuc et al., 1975; Coleman and Burkart, 1977), and adrenalectomy dramatically decreases obesity (Solomon and Mayer, 1973), whereas no effect on basal corticosterone was detected in MC4-R–deficient mice. Furthermore, whereas *ob* and *db* mice are shorter than their wild-type littermates (Heston and Vlahakis, 1962; Wolff, 1963), mice lacking the MC4-R display increased linear growth.

Consistent with a role in body weight regulation, the MC4-R is expressed in a number of hypothalamic sites, including the ventromedial, lateral, dorsomedial, and paraventricular nuclei (Mountjoy et al., 1994), which play an important role in regulating feeding behavior (reviewed in Bray, 1987). These neurons are also in synaptic contact with neurons from the hypothalamic arcuate nucleus and the nucleus of the solitary tract of the brain stem, the primary sites of expression in the brain of POMC (Jacobowitz and O'Donohue, 1978; Watson et al., 1978). Melanocortin peptides derived from processing of POMC, including adrenocorticotrophin (ACTH) and the α-, β- and γ-melanocyte–stimulating hormones (α, β, γ MSH) (reviewed in O'Donohue, 1982), are the sole known ligands for the melanocortin receptors. The observed loss of weight regulation in mice lacking the MC4-R implies an inhibitory role of MC4-R ligand(s) in body energy balance and metabolism. This is further supported by recent pharmacological studies of the cyclic melanocortin analogues, MTII and SHU9119, a high affinity agonist and antagonist, respectively, of both neural melanocortin receptors MC3 and MC4 (Hruby et al., 1995). Intracerebroventricular (ICV) injection of the agonist MTII in four models of hyperphagia (*ob* mice, fasted C57BL/6J mice, A^y mice, and mice coinjected with NPY) suppressed feeding in a dose-dependent manner, and this suppression could be prevented by coinjection of the antagonist SHU9119 (Fan et al., 1997). Furthermore, injection of the antagonist produced a significant increase in food intake. Together with the data presented here, these results demonstrate a novel role for melanocortinergic neurons in tonic inhibition of feeding and metabolism. The increased somatic growth observed in MC4-R–deficient mice also suggests involvement of neurons expressing MC4 receptors in regulation of growth hormone–releasing hormone.

Expression of melanocortin peptides in the brain have previously been implicated in a number of other biological activities, including increasing retention of learned

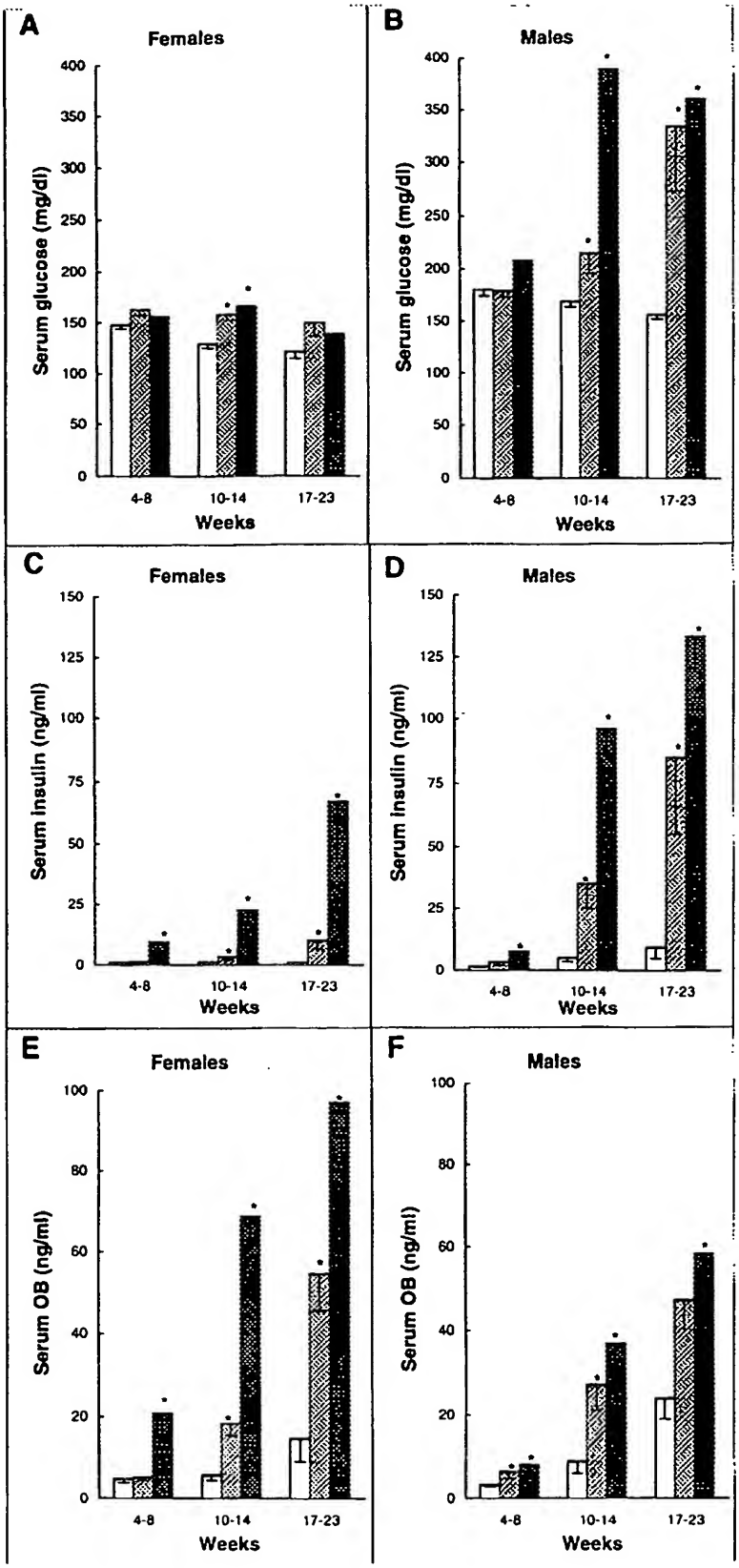


Figure 5. Serum Glucose, Insulin, and Leptin Levels in Mice Lacking the MC4-R

Glucose, insulin, and leptin were each measured on the same serum samples. Open bars represent mean values for wild-type F2 controls, hatched bars represent heterozygotes, and shaded bars represent homozygous mutant mice. Error bars indicate the standard error of the mean. Asterisks denote significant difference ($p < 0.05$ by two-tailed Student t test) compared to control within the same sex and age group. For female mice, the n for wild-type mice at 4–8 weeks, 10–14 weeks, and 17–23 weeks was 11, 14, and 7, respectively; for heterozygotes, 17, 17, and 7, respectively; and for homozygous mutants, 7, 11, and 3, respectively. For male mice, the n for wild-type mice at 4–8 weeks, 10–14 weeks, and 17–23 weeks was 14, 14, and 6, respectively; for heterozygotes, 14, 14, and 5, respectively; and for homozygous mutants, 8, 8, and 9, respectively.

(A and B). Serum glucose levels of female and male mice, respectively. Five μl of serum was analyzed using a glucose oxidase assay.

(C and D). Serum insulin levels of female and male mice, respectively, were assayed by radioimmunoasay using rat insulin as the standard.

(E and F). Serum leptin levels of female and male mice, respectively, were measured by radioimmunoassay.

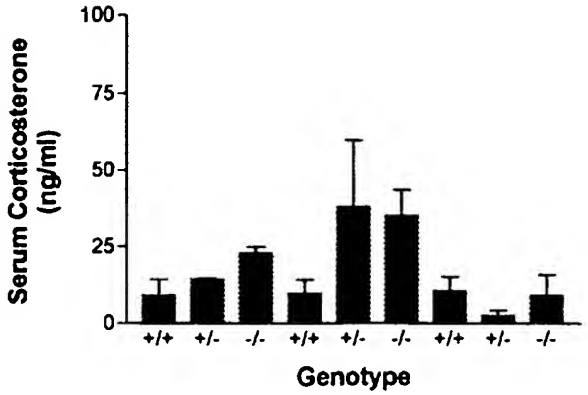


Figure 6. MC4-R Gene Deletion Does Not Affect Basal Serum Corticosterone

Serum corticosterone levels were measured in three sets of sex-matched littermates containing a representative animal of each genotype: +/+ wild-type control, +/− heterozygote, −/− homozygous mutant. Sets are, from left to right, male, female, and male. Males were 15 weeks of age, females were 18 weeks. Data indicate the means of measurements performed using two serum samples obtained on different days. Measurement on each day was performed in duplicate. Bars indicate standard deviation. Analysis of data by two-way ANOVA indicated no significant difference in corticosterone levels as a function of genotype.

behaviors (Murphy and Miller, 1955; DeWied and Jolles, 1982), antipyresis and regulation of temperature control (Lipton and Glyn, 1980; Murphy et al., 1983; Feng et al., 1987), elevation of heart rate, blood pressure, and natriuresis (Klein et al., 1985; Lin et al., 1987). The involvement of MC4-R–dependent signaling in mediating these various biological effects can now be addressed in mice lacking expression of the receptor.

An alternative to the conclusion that signaling by the MC4-R directly influences feeding and metabolism is that MC4-R gene deletion could result in a defect in hypothalamic brain development resulting in hyperphagia and hypometabolism. For example, defects in functioning of the ventromedial hypothalamus in humans and rodents are associated with obesity (reviewed in Bray, 1984). This argument is formally difficult to exclude; however, no gross neuroanatomical defects were observed in brain sections from mice lacking the MC4 receptor. Furthermore, direct evidence for a pharmacological etiology is provided by the modulation of feeding behavior affected by the cyclic melanocortin analogues, MTII and SHU9119, as described above. In addition, the MC4-R–deficient obesity phenotype is strikingly similar to the *agouti* obesity syndrome, consistent with a specific common mode of action of the two mutations (i.e. interference with MC4-R signaling) rather than a developmental defect in MC4-R–deficient mice.

The agouti protein is a pigmentation factor normally expressed in the skin where it regulates the synthesis of pigment by antagonism of the MC1-R on melanocytes. Dominant mutations of the *agouti* locus that result in widespread ectopic expression give rise to the pleiotropic *agouti* obesity syndrome (Bultman et al., 1992; Michaud et al., 1993; Miller et al., 1993; Duhl et al., 1994; Michaud et al., 1994a; 1994b). The demonstration that agouti in vitro is a competitive antagonist of not only the melanocyte MC1-R but also the neural MC4-R led to the hypothesis that agouti induces obesity by chronic antagonism of the MC4-R (Lu et al., 1994). In this study we have observed noteworthy similarities between the MC4-R–deficient and *agouti* obesity syndromes. Mice lacking the MC4-R develop obesity and hyperinsulinemia with a time course very similar to that described for A^y and A^{vy} mice (Dickie and Woolley, 1946; Dickerson and Gowen, 1947; Yen et al., 1976a; Frigeri et al., 1983; Warbritton et al., 1994; reviewed in Yen et al., 1994). The MC4-R–mutant mice also exhibit increased linear growth, a feature unique to the *agouti* syndrome among rodent obesity models. Furthermore, as is also the case for the *agouti* obesity syndrome, the obesity of MC4-R–deficient mice is not dependent on elevation of basal adrenocortical steroids (Wolff and Flack, 1971). Analysis of the expression of neuropeptide Y (NPY, a potent stimulator of feeding) in A^y and MC4-R–deficient mice has uncovered a further common and unique feature of the

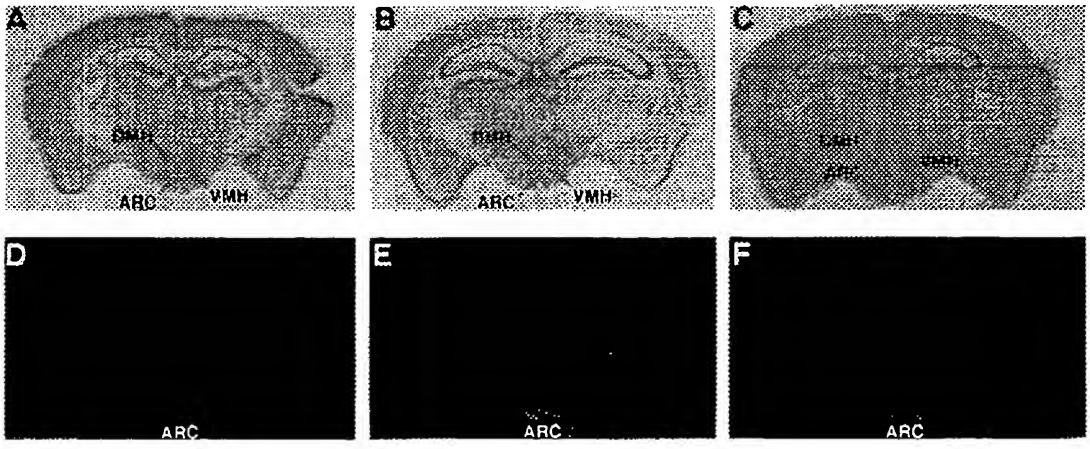


Figure 7. MC4-R Gene Deletion Does Not Affect Brain POMC mRNA Levels

(A–C) Thionin-stained brain sections from wild-type, heterozygous, and homozygous mutant MC4-R–deficient mice, respectively.
(D–F) Autoradiographs of brain sections from wild-type, heterozygous, and homozygous mutant MC4-R–deficient mice, respectively, hybridized with a ^{35}S-POMC antisense cRNA probe.

two melanocortinergic obesity models (R. C. and R. K., personal communication). NPY expression was unchanged in the arcuate nucleus, but dramatically elevated in the dorsal medial hypothalamic (DMH) nucleus in both obese A^y and MC4-R–deficient mice. Elevation of NPY in the arcuate nucleus is found in several obesity models, including *ob* mice (Sanacora et al., 1990; Stephens et al., 1995; Schwartz et al., 1996), but expression of NPY in the DMH has not previously been reported in association with obesity. This finding shows a common change in hypothalamic gene expression in the agouti- and MC4-R–deficient mice, and may imply involvement of the DMH in the melanocortinergic obesity syndrome.

The striking recapitulation of many features of the *agouti* obesity syndrome in MC4-R–deficient mice strongly supports the conclusion that the syndrome derives from agouti-mediated disruption of an MC4-R–dependent signaling pathway involved in the regulation of body weight. Our data are not consistent with an alternative hypothesis that the primary site of agouti action in eliciting the obesity syndrome is peripheral, mediated by elevation of intracellular levels of free calcium in skeletal muscle (Zemel et al., 1995) and/or adipocytes and pancreatic β-cells (Klebig et al., 1995; Manne et al., 1995). Those phenotypic differences we have detected between mice lacking the MC4-R and dominant *agouti* mutants have been quantitative rather than qualitative. For example, the absolute weight gain of MC4-R mutants appears to be greater than that of *agouti* mutants. Keeping in mind that there are considerable variations in the weight of obese yellow mice on different genetic backgrounds and there is no data for body weight of dominant agouti mutants on the 129/Sv × C57BL/6 background of the MC4-R–deficient mice, this weight increase tends to be higher than that reported for age and sex-matched A^y or A^{vy} mice on a variety of backgrounds (Castle, 1941; Dickie and Woolley, 1946; Dickerson and Gowen, 1947; Carpenter and Mayer, 1958; Yen et al., 1976a; Yen et al., 1976b; Frigeri et al., 1983; Wolff, 1987; Frigeri et al., 1988). Similarly, the levels of serum insulin in both males and females homozygous for MC4-R deletion, as well as glucose levels in MC4-R–deficient males, are higher than those reported for age- and sex-matched A^{vy} mice (Frigeri et al., 1983). Lastly, inactivation of the MC4-R results in a greater increase in the length of mice than that observed as a result of ectopic expression of agouti (Castle, 1941; Carpenter and Mayer, 1958). These differences may reflect a dose dependency of MC4-R antagonism on the severity of the obesity syndrome, with deletion of the receptor equivalent to absolute antagonism as opposed to less complete antagonism of the receptor by ectopic agouti expression. This model is supported by 1) data reported here of a gene dosage effect of heterozygosity for receptor deletion on the extent of obesity, hyperinsulinemia, hyperglycemia, and linear growth; by 2) the observation that the level of ectopic agouti expression in agouti transgenics correlates with the severity of obesity, hyperinsulinemia and hyperglycemia (Klebig et al., 1995; Perry et al., 1995); and by 3) dominant agouti mutations resulting from intracisternal A particle (IAP) insertions in which expressivity of the obese phenotype is positively correlated with the levels of agouti expression (Wolff et al., 1986; Duhl et al., 1994; Michaud et al., 1994b). Although agouti is a secreted protein, it acts in a localized fashion and does not appear to circulate at significant levels (Wolff, 1963; Silvers, 1979). Thus, the relevant concentration of agouti for mediating antagonism of the MC4-R is likely that which is expressed in the immediate milieu of the receptor in the brain.

Targeted deletion of the MC4-R has revealed a novel signaling pathway in the brain controlling nutrient intake and energy balance. Further analysis of the regulation and consequences of melanocortinergic signaling should prove instructive in the elucidation of the physiological control of body weight and may provide novel therapies for treatment of obesity. The obesity syndrome resulting from abrogation of MC4-R signaling convincingly mirrors that generated by ectopic agouti expression, demonstrating central antagonism of melanocortin signaling via the MC4-R as the primary cause of the *agouti* obesity syndrome.

Experimental Procedures

Construction of the MC4-R Targeting Vector

Murine MC4-R gene sequences were isolated from a mouse strain 129/Sv genomic phage library (Stratagene) using a probe generated by PCR amplification of human MC4-R coding sequences.

Construction of the MC4-R targeting construct was carried out in the vector pJN2 that was produced in the following manner. The 1.4 kb EcoRI–AvaI fragment of pBR322 was replaced with the synthetic oligonucleotides AAT TAG CGG CCG CAG TAT GCA AAA AAA AGC CCG CTC ATT AGG CGG GCT and CCG AAG CCC GCC TAA TGA GCG GGC TTT TTT TTG CAT ACT GCG GCC GCT. The resulting plasmid, pJN1, was digested with NotI, and the following oligonucleotides were ligated into the NotI site to generate pJN2: GGC CGG CAT GCA TCA AGC TTA TCT CGA GAT CGT CGA CTA CCA TGG TAC ATC GAT CAG GTA CCA TCC CGG GGC and GGC CGC CCC GGG ATG GTA CCT GAT CGA TGT ACC ATG GTA GTC GAC GAT CTC GAG ATA AGC TTG ATG CAT GCC.

A 1.2 kb genomic fragment extending from the HindIII site located approximately 0.6 kb 3′ of the MC4-R gene to the SphI site located approximately 1.8 kb 3′ of the MC4-R gene, representing the 3′ region of genomic homology, was subcloned into SphI–HindIII-digested pJN2. A 3.4 kb genomic fragment extending from the NcoI site located at the 5′ end of the MC4-R gene to the HindIII site located approximately 3.4 kb 5′ of the gene, representing the 5′ region of genomic homology, was also ligated into this plasmid, 5′ of the 1.2 kb HindIII–SphI fragment, and in the same orientation.

The PGK-*neo* expression cassette from the plasmid pKJ1 (Tybulewicz et al. 1991), containing the *neo* gene under the transcriptional control of the mouse phosphoglycerate kinase (PGK-1) promoter and the PGK-1 poly(A) addition site, was ligated between the 5′ and 3′ fragments of MC4-R genomic homology to generate the MC4-R targeting vector. The vector was linearized with NotI digestion prior to electroporation.

ES Cell Transfection

The RF-8 ES cell line (Huang et al., 1996) was cultured on SNL76/7 mitotically inactive feeder cells. For electroporation, cells were trypsinized and resuspended at a concentration of 1.1×10^7/ml in PBS (Ca^{2+}- and Mg^{2+}-free; Gibco). A 0.9 ml aliquot (1×10^7 cells) was mixed with 20 μg of the linearized targeting vector and pulsed at 250V, 500 μF (Bio-Rad Gene Pulser). The cells were then diluted in culture medium, plated at $1–2 \times 10^6$ per 100mm plate containing feeder cells, and placed under selection 24 hr later in G418 sulfate (400 μg/ml powder, Gibco). G418-resistant clones were picked, dissociated with trypsin, and divided into one well each of two 96-well plates. Upon confluence, ES cells were frozen in one of the 96-well plates as described (Ramirez-Solis et al., 1993) and expanded into a 24-well plate from which DNA was prepared, upon confluence, for Southern blot analysis.

Southern Blot Hybridization

Genomic DNA was prepared in situ from ES cells in 24-well plates and from tail biopsies by the procedure of Laird et al. (1991). Approximately 20 μg of genomic DNA was digested with the indicated restriction endonuclease, electrophoresed through a 1% agarose gel, transferred to Hybond N+ membrane (Amersham), and hybridized with the ^{32}P-radiolabeled probes indicated in the text.

Generation of MC4-R–Deficient Mice

The targeted ES clone was injected into C57BL/6J blastocysts as described (Bradley, 1987) to generate chimeras. Male chimeras were bred with C57BL/6J females, and agouti offspring (representing germline transmission of the ES genome) were screened for the presence of the targeted MC4-R gene by Southern blot hybridization of ApaI- and NcoI-digested tail DNA with the flanking probe shown in Figure 1A. Offspring heterozygous for the mutation were bred together, and mice homozygous for the MC4-R deletion were identified by Southern blot hybridization of ApaI- or NcoI-digested tail DNA with the flanking probe.

Weight and Length Measurements

Weight gain was regularly measured, beginning at 3–4 weeks of age, using a Sartorius model #14800 P balance. Length was measured by manual immobilization and extension of the mouse to its full length, always by the same individual, and measurement of the nose-to-anus distance in centimeters.

Food Consumption

Food intake was measured for two *A^y*, two C57BL/6J, four MC4-R homozygous mutants, and four wild-type F2 controls, each housed two to a cage. The mice were housed for at least a week before any measurements were taken. Over a two-week period, a sufficient amount of food for the week was then weighed and provided to the mice ad libitum. Each weekday morning, the remaining food was measured, for a total of eight measurements. Cages were carefully monitored for spillage, which was negligible. The *A^y* and C57BL/6J mice were 9 weeks of age at the time measurement of food consumption was initiated; both the four MC4-R–deficient mice and the F2 controls were each 15, 15, 17.5, and 20.5 weeks of age.

Serum Glucose, Insulin, and Leptin Measurements

For glucose, insulin, and leptin measurements, blood was collected by retroorbital sinus puncture from animals provided with food and water ad libitum. Mice were handled regularly (three times per week for several weeks) prior to bleeding to minimize stress, and cages were singly moved to a separate location at the time of bleeding. For measurement of glucose levels, 5 μl of serum was analyzed in a YSI Model 27 glucose analyzer (Yellow Springs Instrument Company, Inc.) using a glucose oxidase assay. Results are expressed as mg/dl. The range of detection is 0–500 mg/dl, with a coefficient of variation of < 1%. Serum insulin concentration was measured in duplicate in a 10 μl volume by a specific competitive protein binding assay using rat insulin as the standard. Results are expressed as ng/ml. The range of detection is 0.1–25.0 ng/ml with a coefficient of variation of < 10%. Leptin was measured in duplicate in 20 μl of serum using a radioimmunoassay kit to mouse leptin with recombinant mouse leptin as the standard (Linco Research Inc.).

For serum corticosterone measurements, mice were housed at three animals per cage with food and water ad libitum. Male mice were tested at 15 weeks of age, females at 18 weeks. To prevent stress-mediated elevation of corticosterone levels, mice were handled 2–3 times/day for three days prior to drawing blood. Cages were brought one at a time into a separate room, and mice were weighed and then held as if blood were to be drawn. On the fourth day, mice were handled similarly, and blood was drawn between 8:00 and 9:00 A.M. within 30 s of handling. Cages were not returned to the housing room until all of the samples had been obtained. Blood was obtained by snipping the tail tip and collecting blood into a Multivette S Gel tube (Sarstedt). Tubes were placed on ice for 20–40 min and centrifuged for 3–4 min at 14,000 rpm to separate the serum. Two one-μl aliquots of serum from each sample were then assayed for corticosterone levels using an ImmuChem Double Antibody Corticosterone ^{125}I RIA kit (ICN Biomedicals, Inc.).

Histology

For in situ hybridization analysis of POMC gene expression, wild-type heterozygous, and homozygous mutant mice were maintained under a 12 hr light, 12 hr dark cycle at constant temperature. Food (Purina mouse chow) and water were provided ad libitum. Anesthetized (avertin) animals were sacrificed between 1500 and 1700 hr, before lights out via cardiac puncture and perfusion with saline (20 ml) and then 50 ml of ice-cold fixation buffer (4% paraformaldehyde in borate buffer [pH 9.5]). Whole brains were rapidly removed and then postfixed overnight in 10% sucrose/fixative buffer. Blocked hypothalamic sections were frozen in powdered dry ice and then stored at −80°C prior to sectioning.

Antisense POMC probe was prepared by linearizing the plasmid mPOMCE3ribo (kindly provided by Dr. Malcolm Low), containing exon 3 of the mouse POMC gene, with NcoI. [^{35}S] cRNA probes were prepared by transcribing 1 μg of each linearized DNA with T7 DNA polymerase for 1 hr at 37°C as described (Promega). Hypothalamic brain blocks were mounted on a frozen stage and serially sectioned into 4 series of 20 μM slices with a sliding microtome. Sections were prepared and hybridized as previously described (Arriza et al., 1988). Sections were hybridized for 20 hr at 58°C with ^{35}S-labeled probes (5 × 10^6 cpm/ml) in 65% formamide, 0.26 M NaCl, 1.3× Denhardt's solution, 1.3 mM EDTA, 13% dextran sulfate, and 13 mM Tris (pH 8). Sections were then digested with RNase (20 μg/ml) for 30 min at 37°C and then desalted in a series of washes from 4× SSC/1mM DTT to a final stringency of 0.1× SSC/1mM DTT at 65°C for 30 min. Sections were dehydrated in ascending ethanol, vacuum dried at room temperature for 30 min, and then exposed to Dupont Cronex film for several days. Dried slides were then dipped in NTB-2 emulsion (Kodak) and developed after 6 days.

Acknowledgments

Correspondence should be addressed to D. H. The authors would like to thank Bob Tepper and Lou Tartaglia for helpful advice, Paul Paglierani and Steve Ellis for sequencing support, Robert Wurzberger and Renata Tenenbaum for assistance with the leptin and insulin assays, Reginald Riley and Heather MacEachern for animal care, and Lisa DiRocco for genotyping. We would also like to acknowledge the support and helpful discussions from our colleagues at Millennium, Hoffmann-LaRoche, and the Vollum Institute. This work was financially supported by Hoffmann–La Roche, Inc.

Received November 12, 1996; revised December 9, 1996.

References

Arriza, J.L., Simerly, R.B., Swanson, L.W., and Evans, R.M. (1988). The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. Neuron *1*, 887–900.

Baumann, H., Morella, K.K., White, D.W., Dembski, M., Bailon, P.S., Kim, H., Lai, C.F., and Tartaglia, L.A. (1996). The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. Proc. Natl. Acad. Sci. USA *93*, 8374–8378.

Bradley, A. (1987). In Teratocarcinomas and Embryonic Stem Cells, E.J. Robertson, ed. (Oxford, England:IRL Press), pp.113–151.

Bray, G.A. (1984). Hypothalamic and genetic obesity: an appraisal of the autonomic hypothesis and the endocrine hypothesis. Int. J. Obesity *8* (Suppl. 1), 119–137.

Bray, G.A. (1987). Obesity - a disease of nutrient or energy balance? Nutr. Rev. *45*, 33–43.

Bultman, S.J., Michaud, E.J., and Woychik, R.P. (1992). Molecular characterization of the mouse agouti locus. Cell *71*, 1195–1204.

Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R., and Burn, P. (1995). Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science *269*, 546–549.

Campfield, L.A., Smith, F.J., and Burn, P. (1996). The OB protein (leptin) pathway - a link between adipose tissue mass and central neural networks. Horm. Metab. Res. *28*, 619–632.

Carpenter, K.J., and Mayer, J. (1958). Physiologic observations on yellow obesity in the mouse. Am. J. Physiol. *193*, 499–504.

Castle, W.E. (1941). Influence of certain color mutations on body size in mice, rats, and rabbits. Genetics *26*, 177–191.

Chhajlani, V., and Wikberg, J.E.S. (1992). Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. FEBS Lett. *309*, 417–420.

Chhajlani, V., Muceniece, R., and Wikberg, J.E.S. (1993). Molecular cloning of a novel human melanocortin receptor. Biochem. Biophys. Res. Commun. *195*, 866–873.

Chen, H., Charlat, O., Tartaglia, L.A., Woolf, E.A., Weng, X., Ellis, S.J., Lakey, N.D., Culpepper, J., Moore, K.J., Breitbart, R.F., et al. (1996). Evidence that the *diabetes* gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in *db/db* mice. Cell *84*, 491–495.

Chua, S.C., Chung, W.K., Wu-Peng, S., Zhang, Y., Liu, S.M., Tartaglia, L., and Leibel, R.L. (1996). Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. Science *271*, 994–996.

Coleman, D.L., and Burkart, D.L. (1977). Plasma corticosterone concentrations in diabetic (*db*) mice. Diabetologia *13*, 25–26.

DeWied, D., and Jolles, J. (1982). Neuropeptides derived from proopiocortin: behavioral, physiological, and neurochemical effects. Physiol. Rev. *62*, 977–1059.

Dickerson, G.E., and Gowen, J.W. (1947). Hereditary obesity and efficient food utilization in mice. Science *105*, 496–498.

Dickie, M.M., and Woolley, G.W. (1946). The age factor in weight of yellow mice. J. Hered. *37*, 365–368.

Dubuc, P.U., Mobley, P.W., and Mahler, R.J. (1975). Elevated glucocorticoids in obese-hyperglycemic mice. Horm. Metab. Res. *7*, 102.

Duhl, D.M.J., Vrieling, H., Miller, K.A., Wolff, G.L., and Barsh, G.S. (1994). Neomorphic agouti mutations in obese yellow mice. Nat. Genet. *8*, 59–65.

Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J., and Cone, R.D. (1997). Melanocortinergic inhibition of feeding behavior and disruption with an *agouti*-mimetic. Nature, in press.

Feng, J.D., Dao, T., and Lipton, J.M. (1987). Effects of preoptic microinjections of α-MSH on fever and normal temeperature control in rabbits. Brain Res. *18*, 473–477.

Friedman, J.M., and Leibel, R.L. (1992). Tackling a weighty problem. Cell *69*, 217–220.

Frigeri, L.G., Wolff, G.L., and Robel, G. (1983). Impairment of glucose tolerance in yellow (*A^{y}/A*) (BALB/c × VY) F-1 hybrid mice by hyperglycemic peptide(s) from human pituitary glands. Endocrinology *113*, 2097–2105.

Frigeri, L.G., Wolff, G.L., and Teguh, C. (1988). Differential responses of yellow *A^{y}/A* and agouti *A/a* (BALB/c × VY) F1 hybrid mice to the same diets: glucose tolerance, weight gain, and adipocyte cellularity. Int. J. Obesity *12*, 305–320.

Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S.J., DelValle, J., and Yamada, T. (1993a). Molecular cloning of a novel melanocortin receptor. J. Biol. Chem. *268*, 8246–8250.

Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S.J., DelValle, J., and Yamada, T. (1993b). Molecular cloning, expression and gene localization of a fourth melanocortin receptor. J. Biol. Chem. *268*, 15174–15179.

Gantz, I., Shimoto, Y., Konda, Y., Miwa, H., Dickinson, C.J., and Yamada, T. (1994). Molecular cloning, expression, and characterization of a fifth melanocortin receptor. Biochem. Biophys. Res. Comm. *200*, 1214–1220.

Ghilardi, N., Ziegler, S., Wiestner, A., Stoffel, R., Heim, M.H., and Skoda, R.C. (1996). Defective STAT signaling by the leptin receptor in diabetic mice. Proc. Natl. Acad. Sci. USA *93*, 6231–6235.

Griffon, N., Mignon, V., Facchinetti, P., Diaz, J., Schwartz, J.C., and Sokoloff, P. (1994). Molecular cloning and characterization of the rat fifth melanocortin receptor. Biochem. Biophys. Res. Comm. *200*, 1007–1014.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., and Friedman, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. Science *269*, 543–546.

Heston, W.E., and Vlahakis, G. (1962). Genetic obesity and neoplasia. J. Natl. Cancer Inst. *29*, 197–209.

Huang, X.-Z., Wu, J.F., Cass, D., Erle, D.J., Corry, D., Young, S.G., Farese, R.V., and Sheppard, D. (1996). Inactivation of the integrin β6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lungs and skin. J. Cell Biol. *133*, 921–928.

Hruby, V.J., Li, D., Sharma, S.D., Castrucci, A.L., Kesterson, R.A., Al-Obeidi, F.A., Hadley, M.E., and Cone, R.D. (1995). Cyclic lactam α-melanotropin analogues of Ac-Nle^{4}-cyclo [Asp^{5}, D-Phe^{7}, Lys^{10}] α-melanocyte–stimulating hormone-(4-10)-NH^{2} with bulky aromatic amino acids at position 7 show high antagonist potency and selectivity at specific melanocortin receptors. J. Med. Chem. *38*, 3454–3461.

Jacobowitz, D.M., and O'Donohue, T.L. (1978). α-Melanocyte stimulating hormone: immunohistochemical identification and mapping in neurons of rat brain. Proc. Natl. Acad. Sci. USA *75*, 6300–6304.

Johnson, P.R., and Hirsch, J. (1972). Cellularity of adipose depots in six strains of genetically obese mice. J. Lipid Res. *13*, 2–11.

Klebig, M.L., Wilkinson, J.E., Geisler, J.G., and Woychik, R.P. (1995). Ectopic expression of the agouti gene in transgenic mice causes obesity, features of type II diabetes, and yellow fur. Proc. Natl. Acad. Sci. USA *92*, 4728–4732.

Klein, M.C., Hutchins, P.M., Lymangrover, J.R., and Gruber, K.A. (1985). Pressor and cardioaccelerator effects of gamma MSH and related peptides. Life Sci. *36*, 769–775.

Labbe, O., Desarnaud, F., Eggerickx, D., Vassart, G., and Parmentier, M. (1994). Molecular cloning of a mouse melanocortin 5 receptor gene widely expressed in peripheral tissues. Biochemistry *33*, 4543–4549.

Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenisch, R., and Berns, A. (1991). Simplified mammalian DNA isolation procedure. Nucl. Acids Res. *19*, 4293.

Lee, G.W., Proenca, R., Montez, J.M., Carroll, K.M., Darvishzadeh, J.G., Lee, J.I., and Friedman, J.M. (1996). Abnormal splicing of the leptin receptor in *diabetic* mice. Nature *379*, 632–635.

Lin, S.Y., Chaves, C., Wiedemann, E., and Humphreys, M.H. (1987). A γ-melanocyte stimulating hormone-like peptide causes reflex natriuresis after acute unilateral nephrectomy. Hypertension *10*, 619–627.

Lipton, J.M., and Glyn, J.R. (1980) Central administration of peptides alters thermoregulation in the rabbit. Peptides *1*, 15–18.

Low, M.J., Simerly, R.B., and Cone, R.D. (1994). Receptors for the melanocortin peptides in the central nervous system. Curr. Opin. Endocrinol. Diabetes *1*, 79–88.

Lu, D., Willard, D., Patel, I.R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R.P., Wilkison, W.O., and Cone, R.D. (1994). Agouti protein is an antagonist of the melanocyte-stimulating–hormone receptor. Nature *371*, 799–802.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R.E., Lee, G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., Kern, P.A., and Friedman, J.M. (1995). Leptin levels in human and rodent: measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. Nat. Med. *1*, 1155–1161.

Manne, J., Argeson, A.C., and Siracusa, L.D. (1995). Mechanisms for the pleiotropic effects of the agouti gene. Proc. Natl. Acad. Sci. USA *92*, 4721–4724.

Michaud, E.J., Bultman, S.J., Stubbs, L.J., and Woychik, R.P. (1993). The embryonic lethality of homozygous lethal yellow mice (*A^{y}/A^{y}*) is associated with the disruption of a novel RNA-binding protein. Genes Dev. *7*, 1203–1213.

Michaud, E.J., Bultman, S.J., Klebig, M.L., van Vugt, M.J., Stubbs, L.J., Russell, L.B., and Woychik, R.P. (1994a) A molecular model for the genetic and phenotypic characteristics of the mouse lethal yellow (*A^{y}*) mutation. Proc. Natl. Acad. Sci. USA *91*, 2562–2566.

Michaud, E.J., van Vugt, M.J., Bultman, S.J., Sweet, H.O., Davisson, M.T., and Woychik, R.P. (1994b). Differential expression of a new

dominant agouti allele (A^{vy}) is correlated with methylation state and is influenced by parental lineage. Genes Dev. *8*, 1463–1472.

Miller, M.W., Duhl, D.M.J., Vrieling, H., Cordes, S.P., Ollmann, M.M., Winkes, B.M., and Barsh, G.S. (1993). Cloning of the mouse agouti gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. Genes Dev. *7*, 454–467.

Mizuno, T.M., Bergen, H., Funabashi, T., Kleopoulos, S.P., Zhong, Y. G., Bauman, W.A., and Mobbs, C.V. (1996). Obese gene expression: reduction by fasting and stimulation by insulin and glucose in lean mice, and persistent elevation in acquired (diet-induced) and genetic (yellow agouti) obesity. Proc. Natl. Acad. Sci. USA *93*, 3434–3438.

Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., and Cone, R.D. (1992). The cloning of a family of genes that encode the melanocortin receptors. Science *257*, 1248–1251.

Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B., and Cone, R.D. (1994). Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol. Endocrinol. *8*, 1298–1308.

Murphy, M.T., Richards, D.B., and Lipton, J.M. (1983). Antipyretic potency of centrally administered α-melanocyte stimulating hormone. Science *221*, 192–193.

Murphy, J.V., and Miller, R.E. (1955). The effect of adrenocorticotrophic hormone (ACTH) on avoidance conditioning in the rat. J. Comp. Physiol. Psychol. *48*, 47–49.

O'Donohue, T.L. (1982). The opiomelanotropinergic neuronal and endocrine systems. Peptides *3*, 353–395.

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995). Effects of the *obese* gene product on body weight regulation in *ob/ob* mice. Science *269*, 540–543.

Perry, W.L., Hustad, C.M., Swing, D.A., Jenkins, N.A., and Copeland, N.G. (1995). A transgenic mouse assay for agouti protein activity. Genetics *140*, 267–274.

Ramirez-Solis, R., Davis, A.C., and Bradley, A. (1993). Gene targeting in mouse embryonic stem cells. Meth. Enzymol. *225*, 855–878.

Robbins, L.S., Nadeau, J.H., Johnson, K.R., Kelly, M.A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K.G., and Cone, R.D. (1993). Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. Cell *72*, 827–834.

Roselli-Rehfuss, L., Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Low, M.J., Tatro, J.B., Entwistle, M.L., Simerly, R.B., and Cone, R.D. (1993). Identification of a receptor for γ melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. Proc. Natl. Acad. Sci. USA *90*, 8856–8860.

Sanacora, G., Kershaw, M., Finkelstein, J.A., and White, J.D. (1990). Increased hypothalamic content of preproneuropeptide Y messenger ribonucleic acid in genetically obese Zucker rats and its regulation by food deprivation. Endocrinology *127*, 730–737.

Schwartz, M.W., Baskin, D.G., Bukowski, T.R., Kuijper, J.L., Foster, D., Lasser, G., Prunkard, D.E., Porte, D., Woods, S.C., Seeley, R.J., and Weigle, D.S. (1996). Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in *ob/ob* mice. Diabetes *45*, 531–535.

Searle, A.G. (1968). An extension series in the mouse. J. Hered. *59*, 341–342.

Shimizu, H., Shargill, N.S., Bray, G.A., Yen, T.T., and Gesellchen, P.D. (1989). Effects of MSH on food intake, body weight and coat color of the yellow obese mouse. Life Sci. *45*, 543–552.

Silvers, W.K., and Russell, E.S. (1955). An experimental approach to action of genes at the agouti locus in the mouse. J. Exp. Zool. *130*, 199–220.

Silvers, W.K. (1979). The agouti and extension series of alleles, umbrous, and sable. In The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction (New York: Springer-Verlag), pp. 6–44.

Solomon, J., and Mayer, J. (1973). The effect of adrenalectomy on the development of the obese-hyperglycemic syndrome in *OB/OB* mice. Endocrinology *93*, 510–513.

Spiegelman, B.M., and Flier, J.S. (1996). Adipogenesis and obesity: rounding out the big picture. Cell *87*, 377–389.

Stephens, T.W., Basinski, M., Bristow, P.K., Bue-Valleskey, J.M., Burgett, S.G., Craft, L., Hale, J., Hoffmann, J., Hsiung, H.M., Kriauciunas, A., et al. (1995). The role of neuropeptide Y in the antiobesity action of the *obese* gene product. Nature *377*, 530–532.

Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., et al. (1995). Identification and expression cloning of a leptin receptor, OB-R. Cell *83*, 1263–1271.

Tybulewicz, V.L.J., Crawford, C.E., Jackson, P.J., Bronson, R.T., and Mulligan, R.C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-*abl* proto-oncogene. Cell *65*, 1153–1163.

Warbritton, A., Gill, A.M., Yen, T.T., Bucci, T., and Wolff, G.L. (1994). Pancreatic islet cells in preobese yellow $A^{vy}/-$ mice: relation to adult hyperinsulinemia and obesity. Proc. Soc. Exp. Biol. Med. *206*, 145–151.

Watson, S.J., Akil, H., Richard, C.W., and Barchas, J.D. (1978). Evidence for two separate opiate peptide neuronal systems and the coexistence of beta-lipotropin, beta-endorphin, and ACTH immunoreactivities in the same hypothalamic neurons. Nature *275*, 226–228.

Wolff, G.L. (1963). Growth of inbred yellow ($A^y a$) and non-yellow (*aa*) mice in parabiosis. Genetics *48*, 1041–1058.

Wolff, G.L., and Flack, J.D. (1971). Genetic regulation of plasma corticosterone concentration and its response to castration and allogeneic tumour growth in the mouse. Nature New Biol. *232*, 181–182.

Wolff, G.L., Galbraith, D.B., Domon, O.E., and Row, J.M. (1978). Phaeomelanin synthesis and obesity in mice. Interaction of the viable yellow (A^{vy}) and sombre (E^{so}) mutations. J. Hered. *69*, 295–298.

Wolff, G.L., Roberts, D.W., and Galbraith, D.B. (1986). Prenatal determination of obesity, tumor susceptibility and coat color pattern in viable yellow (A^{vy}/a) mice. J. Hered. *77*, 151–158.

Wolff, G.L. (1987). Body weight and cancer. Am. J. Clin. Nutr. *45*, 168–180.

Yen, T.T., Steinmetz, J., and Wolff, G.L. (1970). Lipolysis in genetically obese and diabetes-prone mice. Horm. Metab. Res. *2*, 200–203.

Yen, T.T., Greenberg, M.M., Yu, P.L., and Pearson, D.V. (1976a). An analysis of the relationships among obesity, plasma insulin, and hepatic lipogenic enzymes in "viable yellow obese" mice (A^{vy}/a). Horm. Metab. Res. *8*, 159–166.

Yen, T.T., Allan, J.A., Yu, P.L., Acton, M.A., and Pearson, D.V. (1976b). Triacylglycerol contents and in vivo lipogenesis of *ob/ob*, *db/db* and A^{vy}/a mice. Biochem. Biophys. Acta *441*, 213–220.

Yen, T.Y., Gill, A.M., Frigeri, L.G., Barsh, G.S., and Wolff, G.L. (1994). Obesity, diabetes, and neoplasia in yellow $A^{vy}/-$ mice: ectopic expression of the agouti gene. The FASEB J. *8*, 479–488.

Zemel, M.B., Kim, J.H., Woychik, R.P., Michaud, E.J., Kadwell, S.H., Patel, I.R., and Wilkison, W.O. (1995). Agouti regulation of intracellular calcium: role in the insulin resistance of viable yellow mice. Proc. Natl. Acad. Sci. USA *92*, 4733–4737.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. Nature *372*, 425–431.

Antagonism of the Melanocortin System Reduces Cold and Mechanical Allodynia in Mononeuropathic Rats

Dorien H. Vrinten,[1,2] Willem Hendrik Gispen,[1] Gerbrand J. Groen,[2] and Roger A. H. Adan[1]

Departments of [1]Medical Pharmacology and [2]Anesthesiology, Rudolf Magnus Institute for Neurosciences, University Medical Centre Utrecht, 3584 CG Utrecht, The Netherlands

The presence of both pro-opiomelanocortin-derived peptides and melanocortin (MC) receptors in nociception-associated areas in the spinal cord suggests that, at the spinal level, the MC system might be involved in nociceptive transmission. In the present study, we demonstrate that a chronic constriction injury (CCI) to the rat sciatic nerve, a lesion that produces neuropathic pain, results in changes in the spinal cord MC system, as shown by an increased binding of ^{125}I-NDP-MSH to the dorsal horn. Furthermore, we investigated whether intrathecal administration (in the cisterna magna) of selective MC receptor ligands can affect the mechanical and cold allodynia associated with the CCI. Mechanical and cold allodynia were assessed by measuring withdrawal responses of the affected limb to von Frey filaments and withdrawal latencies upon immersion in a 4.5°C water bath, respectively. We show that treatment with the MC receptor antagonist SHU9119 has a profound anti-allodynic effect, suggesting that the endogenous MC system has a tonic effect on nociception. In contrast, administration of the MC4 receptor agonists MTII and D-Tyr-MTII primarily increases the sensitivity to mechanical and cold stimulation. No antinociceptive action was observed after administration of the selective MC3 receptor agonist Nle-γ-MSH. Together, our data suggest that the spinal cord MC system is involved in neuropathic pain and that the effects of MC receptor ligands on the responses to painful stimuli are exerted through the MC4 receptor. In conclusion, antagonism of the spinal melanocortin system might provide a new approach in the treatment of neuropathic pain.

Key words: neuropathic pain; chronic constriction injury; allodynia; melanocortins; melanocortin-4 receptor; spinal cord; dorsal horn; in situ *^{125}I-NDP-MSH binding*

In humans, damage to the nervous system (a peripheral nerve, dorsal root ganglion, dorsal root, or the CNS) can lead to a pain state referred to as neuropathic pain. This syndrome is characterized by spontaneous pain in combination with allodynia (pain evoked by normally nonpainful stimuli) and hyperalgesia (an increased response to painful stimuli). In current clinical practice, several drugs are used to control neuropathic pain, including tricyclic antidepressants (for review, see Ollat and Cesaro, 1995; Kingery, 1997), anticonvulsants (Rosenberg et al., 1997), systemic administration of local anesthetics (Glazer and Portenoy, 1991; Rowbotham et al., 1991), and NMDA receptor antagonists (Backonja et al., 1994; Felsby et al., 1996). Despite this wide range of drugs, the treatment of neuropathic pain is often unsatisfactory and limited by the occurrence of adverse side effects.

Over the past decade, a number of animal models of neuropathic pain have become available, producing symptoms that closely resemble those observed in human neuropathic pain. Research using these preclinical models has yielded an array of potential new analgesics, including different enzyme inhibitors, ion channel blockers, and ligands for various receptors (for review, see Chizh et al., 1999; Yaksh, 1999). Another potential target in the control of pain that has received very little attention is the melanocortin (MC) system. It has been reported previously that central administration of the melanocortins adrenocorticotropic hormone (ACTH) and α-melanocyte stimulating hormone (α-MSH) cause hyperalgesia in various pain tests (Bertolini et al., 1979; Sandman and Kastin, 1981; Williams et al., 1986). Furthermore, these peptides have also been shown to antagonize the analgesic effects of morphine and β-endorphin (Gispen et al., 1976; Wiegant et al., 1977; Smock and Fields, 1981). The mechanisms through which these effects were exerted, however, remained unclear because no receptors for these peptides were identified. Only in recent years, five MC receptors (MC-Rs) subtypes have been identified (for review, see Cone et al., 1996; Tatro, 1996), of which the MC3 and MC4 receptors are expressed in the nervous system. Compared with the MC3 receptor, the MC4 receptor has a much more widespread distribution throughout the brain. Moreover, it is the only subtype of which expression has been demonstrated in the spinal cord (Mountjoy et al., 1994). Binding of ^{125}I-NDP-MSH, a synthetic α-MSH analog, to rat spinal cord demonstrated that the most abundant MC receptor expression is present in the superficial dorsal horn (lamina I and II) and in the gray matter surrounding the central canal (lamina X), areas that are important in nociceptive transmission (van der Kraan et al., 1999). Furthermore, pro-opiomelanocortin (POMC) mRNA was also demonstrated in spinal cord (van der Kraan et al., 1999), and immunoreactivity for the POMC-derived peptides β-endorphin, ACTH, and α-MSH has been described in the dorsal horn and lamina X (Tsou et al., 1986; Plantinga et al., 1992). Together, these findings suggest the presence of a functional MC system in the rat spinal cord. Considering the localization of ^{125}I-NDP-MSH binding in nociception-associated areas in the spinal cord and the fact that the MC4 receptor is the only MC receptor subtype for which mRNA has been detected in the spinal cord, the spinal MC4 receptor might be a potential target in the ongoing search for new analgesics.

As recently selective ligands for the MC receptors became available, it is now possible to study a putative role for the MC4 receptor in the control of neuropathic pain. The aim of the present study was to investigate whether changes in the spinal cord MC system play a role in neuropathic pain. Therefore, *in situ* binding of ^{125}I-NDP-MSH to rat lumbar spinal cord sections was quantified. The chronic constriction injury (CCI) (Bennett and Xie, 1988) was chosen because of its wide acceptance as a reliable and reproducible model for neuropathic pain. In addition, we investigated whether selective MC receptor ligands can alter the response of control and mononeuropathic rats to painful stimuli.

We demonstrate that a CCI results in an increase in ^{125}I-NDP-

Received April 6, 2000; revised July 6, 2000; accepted Aug. 9, 2000.

We thank Simone Duis, Nienke Wanders, and Jan Brakkee for technical assistance on the *in vivo* experiments, and Keith Garner for performing the ^{125}I-NDP-MSH *in situ* binding assay.

Correspondence should be addressed to Dr. Roger A. H. Adan, Department of Medical Pharmacology, Rudolf Magnus Institute for Neurosciences, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands. E-mail: adan@med.uu.nl.

 0270-6474/00/208131-07$15.00/0

Table 1. Affinity (Ki) and potency (EC_{50}) of melanocortin receptor ligands for the rat MC3 and MC4 receptors

	Rat MC3		Rat MC4	
Ligand	Ki (nM)	EC_{50} (nM)	Ki (nM)	EC_{50} (nM)
MTII	4.77 ± 2.13	0.78 ± 0.17	1.74 ± 0.77	0.01 ± 0.004
D-Tyr-MTII	204 ± 87.2	20.3 ± 7.1	3.84 ± 0.84	0.47 ± 0.19
Nle-γ-MSH	1.44 ± 0.26	1.26 ± 0.10	77.5 ± 37.7	11.0 ± 3.92
SHU9119	0.879 ± 0.170		0.238 ± 0.060	

Affinities and potencies are determined on HEK 293 cells expressing either the rat MC3 or the rat MC4 receptor using ^{125}I-NDP-MSH as radioligand (for Ki) or the *LacZ* reporter gene (for EC_{50}). Data are expressed as mean ± 95% confidence interval (adapted from Adan et al., 1999).

MSH binding to the spinal cord, suggesting an increase in MC receptor levels. We also show that, in CCI rats, intrathecal administration of the MC receptor antagonist SHU9119 induced a decreased sensitivity to cold and mechanical stimulation, whereas the strong MC receptor agonist MTII or the more selective MC4 receptor agonist D-Tyr MTII had the opposite effect. In contrast, in control rats, these ligands had no effect on sensitivity. Furthermore, we show that treatment with the selective MC3-R agonist Nle-γ-MSH had no effect on sensitivity.

MATERIALS AND METHODS

Peptides

For *in vivo* administration, MTII [Melanotan-II or cyclo-[Nle^4, Asp^5, D-Phe^7, Lys^{10}]α-MSH-(4–10)], SHU9119 [cyclo-[Nle^4, Asp^5, D-Nal(2)^7, Lys^{10}]α-MSH-(4–10)], D-Tyr-MTII (cyclo-[Nle^4, Asp^5, D-Tyr^7, Lys^{10}]α-MSH-(4–10)], and Nle-γ-MSH (Ac-[Nle^3]-γ_2-MSH-NH_2) were used. MTII was purchased from Bachem Feinchemicalien (Buberdorf, Switzerland), and SHU9119, Nle-γ-MSH, and D-Tyr-MTII were synthesized using g-fluorenyl methoxycarbonyl solid phase synthesis as reported previously (Schaaper et al., 1998). Peptides were purified using reverse-phase preparative HPLC to a purity of ±90%, estimated after analysis by analytical HPLC at 215 nm. Molecular weight was confirmed by mass spectrometry performed on a Micromass Quattro single quadrupole. Potencies and affinities of these four peptides for the rat MC3 and MC4 receptor are shown in Table 1.

For *in situ* melanocortin binding to spinal cord cryosections, ^{125}I-NDP-MSH was used. NDP-MSH (Melanotan-I or [Nle^4, D-Phe^7]α-MSH) was purchased from Bachem Feinchemicalien and iodinated using bovine lacto-peroxidase (Calbiochem, Lucerne, Switzerland) and ^{125}I-Na (ICN Biochemicals, Costa Mesa, CA) as described previously (Huang et al., 1997), followed by HPLC purification on a C18 column (μBondapak 3.9 × 300 mm; Waters, Milford, MA).

Animals

Fifty-nine male Wistar rats weighing 200–240 gm at the start of the study were used. Animals were housed in groups of two to three in plastic cages on a sawdust bedding. They were kept at a 12 hr light/dark cycle, with food and water available *ad libitum*. All testing procedures in this study were performed according to the Ethical Guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and were approved of by the Ethics Committee on Animal Experiments of the Utrecht University.

Surgery

Animals were anesthetized with a single subcutaneous injection of Hypnorm (Janssen Pharmaceutical Ltd., Grove, Oxford, UK) containing 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone, at a dose of 0.3 ml/kg bodyweight.

In 33 animals, the right sciatic nerve was exposed at midthigh level by blunt dissection, and a CCI was made by placing four loose ligatures of 4-0 chromic catgut (Ethicon, Norderstedt, Germany) around the nerve, as described previously by Bennett and Xie (1988). In four animals, the same procedure was performed except for placement of the ligatures (sham surgery). After this, the incision was closed with silk sutures, and the animals were allowed to recover. The remaining 22 animals only received a cisterna magna cannula (control animals).

Placement of the cannulas was performed 2 weeks after the sham or CCI lesion. Rats were again anesthetized and placed in a stereotactic frame. The skull was exposed by a midline incision. A steel cisterna magna cannula was inserted through a burr hole just before the squama occipitalis, and two small screws were placed lateral to the midline for extra fixation. Cannula and screws were fastened with dental acrylic. The animals were allowed a 4 d recovery period before testing was initiated.

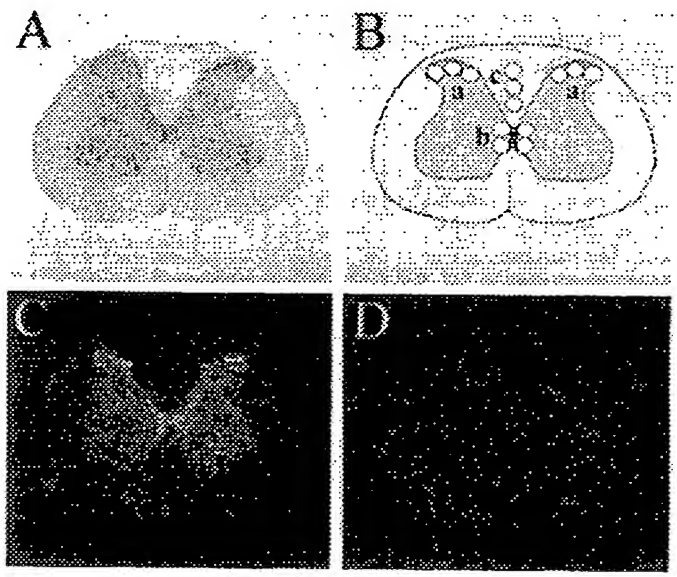


Figure 1. ^{125}I-NDP-MSH binding to rat spinal cord sections. *A*, Nissl staining of a representative spinal cord section, demonstrating the neuroanatomy. *B*, Diagram representing the sampling template used for determining ^{125}I-NDP-MSH binding in x-ray film autoradiograms of rat spinal cord sections. *a*, Superficial dorsal horn (left and right); *b*, lamina X; *c*, dorsal white matter column used for determining background value. For each region, the mean value of three (superficial dorsal horn and background) or four (lamina X) samples was calculated. *C*, *D*, x-Ray film autoradiogram of ^{125}I-NDP-MSH binding to a representative rat spinal cord section. Sections were incubated with ^{125}I-NDP-MSH in the absence (*C*) or presence (*D*) of 3 μM non-iodinated NDP-MSH. Specificity of binding present in *C* is demonstrated by its inhibition in *D*.

In situ *^{125}I-NDP-MSH binding to spinal cord*

Seven animals that remained naive to treatment (four sham and three CCI animals) were used for *in situ* ^{125}I-NDP-MSH binding.

Tissue preparation. Four weeks after placement of the ligatures or sham surgery, the rats were killed by decapitation. The lumbar spinal cord was rapidly removed and frozen by submersion in 2-methyl-butane (Fluka Chemika, Buchs, Switzerland) on dry ice. Spinal cords were stored at −80°C until further processing. From lumbar segments L4–L6, cryostat sections (16 μm) were prepared and mounted on gelatin-coated slides (two sections from each segment per slide).

In situ *binding assay.* From each animal, one slide was incubated with ^{125}I-NDP-MSH as described previously (Tatro, 1993). In short, the sections were prewashed, incubated with ^{125}I-NDP-MSH (10^6 cpm/ml) in binding buffer for 1 hr, washed six times to stop binding reactions, and rapid air dried. A second slide from each animal was incubated with ^{125}I-NDP-MSH in the presence of 3 μM non-iodinated NDP-MSH to determine the specificity of tracer binding. All binding assays were done on the same day, in one experimental session.

To visualize the neuroanatomy more clearly, an adjacent section was Nissl stained (Fig. 1*A*).

Autoradiography and analysis. Autoradiography was performed by exposing an x-ray film (BioMax MR; Eastman Kodak, Rochester, NY) directly to the slides for 1 week. All slides were run on the same, single film, with CCI and sham samples randomly divided over the film. Autoradiograms were digitized and quantitatively analyzed using the MCID

(Microcomputer Imaging Device; Imaging Research Inc., St. Catharines, Ontario, Canada). For each section, binding was measured in three anatomic regions, using a sampling template as depicted in Figure 1*B*. Within each region, three or four samples were measured, and the mean value was calculated. Specific binding was calculated by subtraction of the mean background value, determined within the dorsal white matter column of the same section. Absorbance values were converted into counts per minute using a linear calibration curve.

Drug administration

Thirty CCI and 22 control animals were used to study the effects of the different peptides on nociception. Peptides were dissolved in 10 μl of saline and injected through the cisterna magna cannula by means of a Hamilton syringe.

On each testing day, CCI rats were randomly divided into three groups (n = 10 each), each group randomly and blindly receiving one of the following doses: vehicle, SHU9119, 0.15, 0.5, or 1.5 μg (0.140, 0.466, or 1.40 nmol, respectively); MTII, 15, 30, 100, or 500 ng (14.6, 29.2, 97.6, or 488.2 pmol, respectively); D-Tyr-MTII, 0.3, 1.0, or 3.0 μg (0.289, 0.962, or 2.885 nmol, respectively); Nle-γ-MSH, 5 μg (3.22 nmol) or a combination of 15 ng of MTII and 0.5 μg of SHU9119. Thus, in total, 13 groups of 10 CCI animals were tested.

Similarly, on each testing day, control rats were randomly divided in two groups (n = 11 each), each group randomly and blindly receiving one of the following doses: vehicle, 1.5 μg of SHU9119, 500 ng of MTII, or 3 μg of D-Tyr-MTII (corresponding to the highest doses tested in CCI animals). Thus, in total, four groups of 11 control animals were tested.

Using this experimental setup, animals received only a single injection with a single dose on each testing day. The study was continued until all doses of all drugs were tested. Animals were given at least 2 d rest between drug injections to minimize any possibility of drug interactions or development of tolerance.

Testing procedures

Temperature stimulation test. Withdrawal latency to a temperature stimulus was measured by immersing the right (experimental) hind paw into a 4.5 or 47.5°C water bath. Upon immersion of the paw, an electronic circuit including a timer was closed. Withdrawal of the paw resulted in a discontinuation of the circuit, which stopped the timer, thus allowing a precise registration of the withdrawal latency time. Cutoff time for both temperatures was set at 10 sec to avoid skin damage.

Mechanical stimulation test. Foot withdrawal threshold in response to a mechanical stimulus was determined using a series of von Frey filaments (Stoelting, Wood Dale, IL), ranging from 1.08 to 21.09 gm. Animals were placed in a plastic cage with a metal mesh floor, allowing them to move freely. They were allowed to acclimatize to this environment before the experiment. The filaments were presented to the midplantar surface as described by Chaplan et al. (1994), starting with the smallest filament. Each probe was applied to the foot until it just bent, and the smallest filament eliciting a foot withdrawal response was considered the threshold stimulus.

For both mechanical and temperature stimulation tests, baseline values were determined, and measurements were repeated 15, 30, and 60 min after drug or vehicle administration.

Grooming assay

In eight CCI animals, a grooming assay was performed as described by Gispen et al. (1975). In short, animals were placed in a plastic observation cage immediately after injection of 500 ng of MTII (n = 4) or saline (n = 4) through the cisterna magna cannula. Starting 10 min after injection, grooming (face washing, genital grooming, body licking and grooming, and scratching and paw licking) was scored every 15 sec. Observation was stopped 50 min after injection.

Data analysis

All data are expressed as mean ± SEM for visualization purposes only.

For *in situ* ^{125}I-NDP-MSH binding to spinal cord, the overall mean of levels L4–L6 and one to two sections per rat (thus rendering one data point per anatomic region per rat) were used to calculate group means and SEM. Differences between sham and CCI groups were analyzed using an independent Student's t test.

For the temperature stimulation test, the difference between baseline and postinjection withdrawal latency was calculated for each animal at each time point.

To obtain a linear scale of perceived intensity in the mechanical stimulation test, the logarithm of the withdrawal thresholds was plotted. As for the temperature stimulation test, differences between post-treatment and pretreatment withdrawal thresholds were calculated.

For mechanical and temperature stimulation, differences in baseline values between control and CCI groups and differences between drug treatment groups were analyzed using the Kruskall–Wallis test because of the nonparametric nature of the data. When appropriate, *post hoc* analysis was performed using the Mann–Whitney U test, comparing each treatment dose with vehicle and for each treatment comparing the highest dose with the intermediate and lowest dose, respectively. A Bonferroni correction was performed.

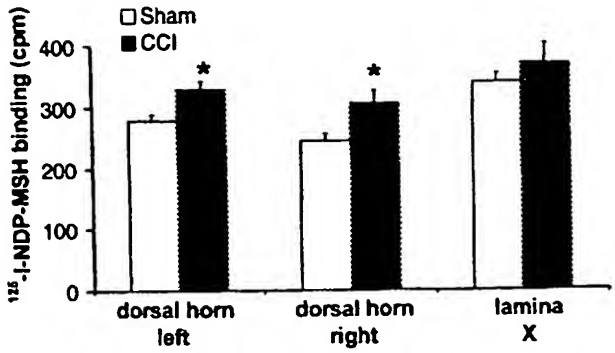


Figure 2. ^{125}I-NDP-MSH binding levels in different anatomic regions in rat lumbar spinal cord (L4–L6) cryostat sections. Spinal cords were collected 4 weeks after CCI or sham surgery. Regions were analyzed by sampling the corresponding region of an x-ray film autoradiogram, using a template as depicted in Figure 1*A*. Values were converted to counts per minute using a linear calibration curve. Specific binding within each region was determined by subtracting the mean background value obtained from the dorsal white matter from the same section. For each region, the overall mean of levels L4–L6 from one or two sections was calculated per rat. Data are represented as mean ± SEM of four (sham) or three (CCI) rats. $^*p <$ 0.05 versus sham.

Where possible, dose–response curves were generated. Therefore, postinjection values were expressed as a percentage of baseline value. Mean ± SEM of these percentages were plotted against the administered dose. Dose–response curves are reported for the time of peak effect (30 min after injection for MTII and D-Tyr-MTII, and 15 min after injection for SHU9119).

Differences in grooming scores were analyzed using an independent Student's t test. For all tests, a probability level of $p \leq 0.05$ was the criterion for a significant difference.

RESULTS

In situ ^{125}I-NDP-MSH binding to spinal cord

Specificity of the ^{125}I-NDP-MSH binding is indicated by its inhibition in the presence of 3 μM NDP-MSH, which reduced binding to background level (Fig. 1*C*,*D*).

As demonstrated previously (van der Kraan et al., 1999), specific ^{125}I-NDP-MSH binding was highest in the superficial dorsal horn (corresponding to lamina I–II) and lamina X. In CCI animals, binding in lamina I–II on both the ipsilateral and contralateral sides was significantly increased compared with sham animals (125.5 and 118.7% of sham values, respectively). In contrast, binding to lamina X did not differ between groups (Fig. 2).

Baseline values for temperature and mechanical stimulation

In control animals, the mean baseline mechanical withdrawal threshold for all four groups at all time points was 21.09 ± 0 gm (mean ± SEM). In CCI animals, the overall mean baseline was significantly lower (5.32 ± 0.21 gm, ranging from 4.83 ± 0.50 to 6.67 ± 1.06 gm for the 13 different randomized groups), thus indicating a mechanical allodynia.

At 4.5°C, overall mean baseline withdrawal latency in control animals was 9.86 ± 0.06 sec (ranging from 9.75 ± 0.19 to 10 ± 0 sec). In CCI animals, mean baseline withdrawal latency was significantly lower (6.05 ± 0.35 sec, ranging from 3.47 ± 0.41 to 7.36 ± 1.07 sec), thus demonstrating a cold allodynia.

At 47.5°C, overall mean baseline withdrawal latency in control animals was 4.52 ± 0.22 sec (ranging from 3.57 ± 0.26 to 5.47 ± 0.45 sec). This value was not significantly different from that in CCI animals (4.66 ± 0.22 sec, ranging from 3.47 ± 0.41 to 6.98 ± 1.26 sec). These data are shown in Figure 3.

Although baseline values could differ between randomized groups, there was no correlation between these values and administration of either an agonist or antagonist, nor were baseline values consistently changed by previous administration of either an agonist or antagonist (data not shown).

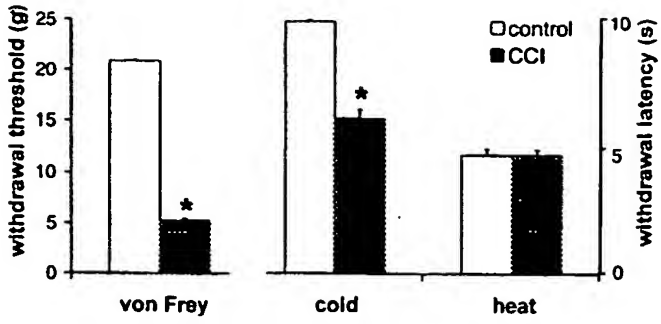


Figure 3. Baseline withdrawal thresholds to von Frey stimulation and baseline withdrawal latencies to cold stimulation (4.5°C) and heat stimulation (47.5°C) in control and neuropathic rats. Data are presented as mean ± SEM of 13 groups of 10 rats (CCI) or 4 groups of 11 rats each (control). $^*p < 0.05$.

Vehicle injection

An injection of 10 μl of saline through the cisterna magna cannula had no effect on the responses to any of the tests performed, in neither CCI rats nor control rats.

Administration of SHU9119

In CCI rats, treatment with SHU9119 (0.15, 0.5, and 1.5 μg) produced a tactile anti-allodynic effect, as shown by a dose-dependent increase in withdrawal thresholds to von Frey stimulation (Fig. 4*A*) compared with vehicle treatment. Peak effects were reached 15 min after injection, resulting in a withdrawal threshold of up to 170.5 ± 7.25% of baseline value (mean ± SEM) with 1.5 μg of SHU9119 (Fig. 5*A*).

As for the mechanical withdrawal thresholds, withdrawal latencies to cold stimulation also increased upon administration of SHU9119 (Fig. 6*A*). The cold anti-allodynic effect of the two lowest doses of SHU9119 showed a dose-dependency as observed for the tactile anti-allodynic effect. However, the highest dose tested (1.5 μg) only produced a small increase in withdrawal latencies. This group consisted of only four animals, and baseline withdrawal latencies of two of these four animals were already at cutoff value, leaving no room for a further increase in latency. In the remaining two animals latencies, however, did increase to cutoff value in one case and to 174% of baseline in the other case. Treatment with SHU9119 did not cause any changes in withdrawal latencies at 47.5°C (data not shown).

In control rats, administration of 1.5 μg of SHU9119 had no effect on responses to mechanical, cold, or heat stimulation (data not shown).

Administration of MTII and D-Tyr-MTII

In CCI rats, administration of the MC receptor agonist MTII (15, 30, 100, and 500 ng) produced a dose-dependent decrease in withdrawal thresholds to mechanical stimulation (Fig. 4*B*). Thirty minutes after injection, withdrawal thresholds were reduced to 4.64 ± 0.52% of baseline (mean ± SEM) with the highest dose tested (Fig. 5*B*).

Similarly as for tactile thresholds, MTII dose-dependently decreased withdrawal latencies at 4.5°C (Fig. 6*B*). The most potent effect was observed with the highest dose tested, which reduced latencies to 9.68 ± 5.07% (mean ± SEM) of baseline value (Fig. 7). As for SHU9119, treatment with MTII caused no significant changes in withdrawal latency to a heat stimulus.

Administration of the more selective MC4 receptor agonist D-Tyr-MTII produced similar results as those observed with MTII, with ~10 times higher doses (0.3, 1, and 3 μg) resulting in a dose-dependent decrease in withdrawal thresholds to von Frey stimulation (Fig. 4*C*) and in withdrawal latencies to cold stimulation (Fig. 6*C*). Values were decreased to 14.73 ± 6.04 and 20.04 ± 5.14% (mean ± SEM) of baseline values, respectively (Figs. 5*B*, 7). As for MTII, administration of D-Tyr-MTII had no effect on withdrawal latencies at 47.5°C.

In control rats, the highest dose of both ligands (500 ng of MTII or 3 μg of D-Tyr-MTII) did not cause any changes in responses to mechanical, cold, or heat stimulation (data not shown).

Coadministration of MTII and SHU9119

Coadministration of 15 ng of MTII, a dose which by itself had no effect on sensory thresholds (Figs. 4*B*, 6*B*), and 0.5 μg of SHU9119 in CCI rats resulted in a complete inhibition of the cold and mechanical anti-allodynic effect of SHU9119 (data not shown).

Administration of Nle-γ-MSH

In CCI rats, a single, high dose (5 μg) of the selective MC3 agonist Nle-γ-MSH was tested. No decreased or increased response was observed to either mechanical or thermal stimulation (data not shown).

In Figure 8, a summary of the described effects of the different MC receptor ligands is presented.

Grooming behavior

Total grooming scores after injection of saline or 500 ng of MTII into the cisterna magna were 50.67 ± 6.39 and 54.5 ± 14.5, respectively (mean ± SEM). These values were not significantly different (data not shown).

DISCUSSION

In the spinal cord, the expression of the MC4 receptor overlaps with that of the POMC-derived peptides α-MSH and ACTH (Tsou et al., 1986; van der Kraan et al., 1999) in nociception-associated areas. Therefore, we hypothesized that, at the spinal level, the MC system is involved in the processing of nociceptive information. Here we show for the first time that changes in the spinal cord MC system occur after a CCI to the rat sciatic nerve, a lesion that causes neuropathic pain. As shown in Figure 2, *in situ* binding of the synthetic MC receptor ligand ^{125}I-NDP-MSH is increased in lumbar (L4–L6, corresponding with sciatic nerve input) spinal cord

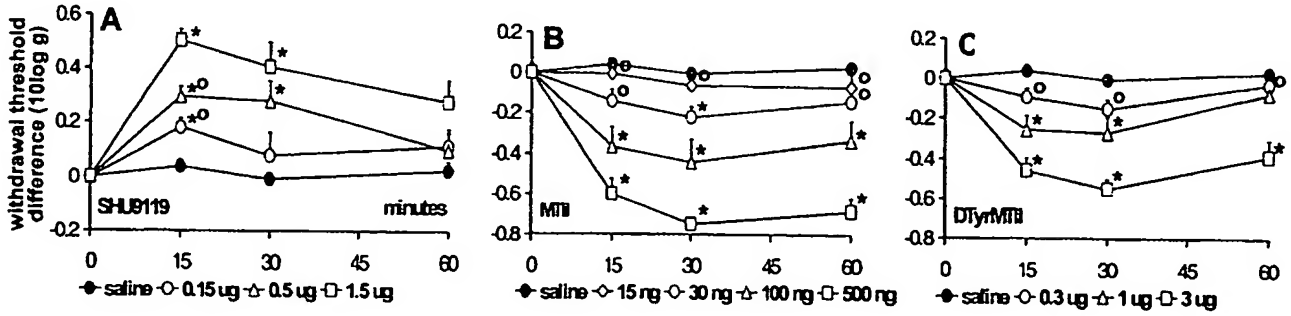


Figure 4. The effect of intrathecal SHU9119 (*A*), MTII (*B*), and D-Tyr-MTII (*C*) on withdrawal thresholds to von Frey stimulation in neuropathic rats. Thresholds are transformed to the logarithm of the applied force. Differences between postinjection and preinjection (baseline) values are plotted. Data are presented as mean ± SEM of 10 rats each, except 1.5 μg of SHU9119 ($n = 4$). $^*p < 0.05$ versus vehicle; $^\circ p < 0.05$ versus highest dose of MTII, D-Tyr-MTII, or SHU9119.

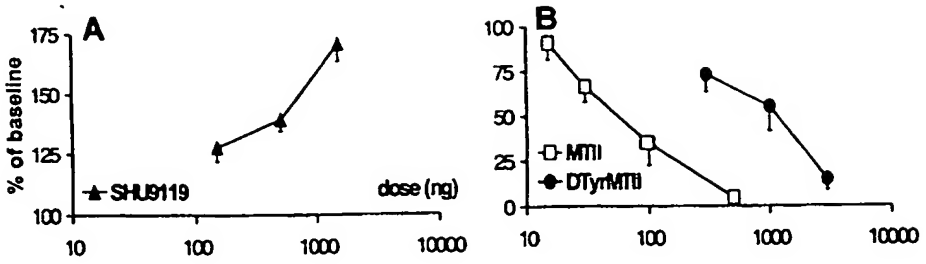


Figure 5. Dose–response curves of the effect of intrathecal SHU9119 (*A*), MTII (*B*), and D-Tyr-MTII (*B*) on von Frey withdrawal thresholds in neuropathic rats. Values represent threshold at 30 min after injection as a percentage of baseline threshold. Data are presented as mean ± SEM of 10 rats each, except 1.5 μg of SHU9119 ($n = 4$).

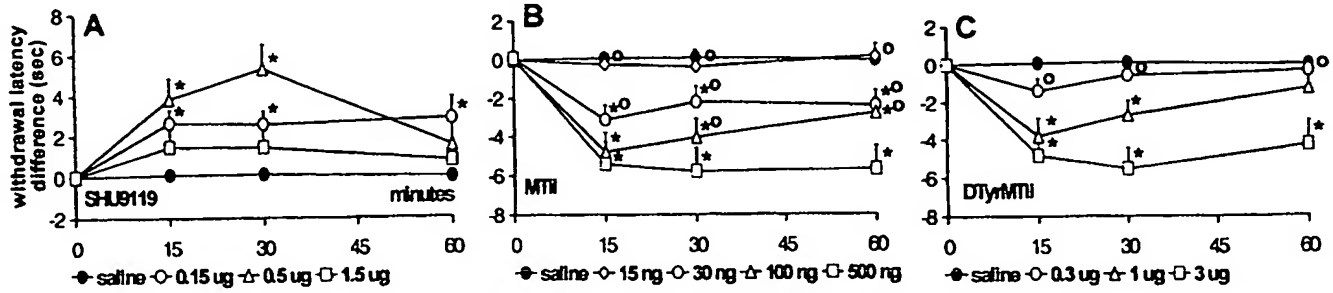


Figure 6. The effect of intrathecal SHU9119 (*A*), MTII (*B*), and D-Tyr-MTII (*C*) on withdrawal latencies to cold stimulation (4.5°C) in neuropathic rats. Differences between postinjection and preinjection (baseline) values are plotted. Data are presented as mean ± SEM of 10 rats each, except 1.5 μg of SHU9119 ($n = 4$). $^*p < 0.05$ versus vehicle; $^\circ p < 0.05$ versus highest dose of MTII, D-Tyr-MTII, or SHU9119.

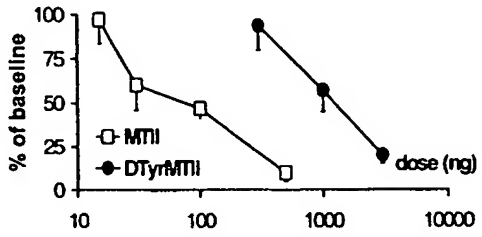


Figure 7. Dose–response curves of the effect of intrathecal MTII and D-Tyr-MTII on withdrawal latencies to cold stimulation (4.5°C) in neuropathic rats. Values represent threshold at 30 min after injection as a percentage of baseline threshold. Data are presented as mean ± SEM of 10 rats each.

sections of CCI rats compared with sham operated animals, suggesting an upregulation of spinal cord MC receptors. It is not likely that these changes are caused by the profound deafferentation associated with a CCI lesion per se (Basbaum et al., 1991; Carlton et al., 1991), because van der Kraan et al. (1999) have shown that crushing the sciatic nerve, another lesion producing extensive nerve fiber loss, did not lead to significant differences in ^{125}I-NDP-MSH binding levels compared with sham surgery.

Of the anatomic regions we investigated, which were the regions with the highest intensity of binding, the superficial dorsal horn both ipsilateral and contralateral to the lesion showed this increased binding. Bilateral changes associated with CCI have been described for other systems as well, including opioid binding sites (Stevens et al., 1991), calcitonin gene-related peptide and substance P immunoreactivity (Kajander and Xu, 1995), metabolic and nitric oxide synthase activity (Mao et al., 1992); (Choi et al., 1996), and transsynaptic degeneration (Hama et al., 1994). The contralateral changes might be explained by changes in primary afferents that cross the midline, commissural connections between intrinsic spinal neurons (Sugimoto et al., 1990), or descending control systems affecting both sides of the spinal cord (Besse et al., 1992).

The superficial dorsal horn, the area that displayed an increased ^{125}I-NDP-MSH binding in our experiments, corresponds with the predominant entry zone of cutaneous fine diameter primary afferents of the sciatic nerve. In contrast, the gray matter surrounding the central canal, an area that receives mostly visceral input, showed no differences in binding. These findings suggest that changes in the endogenous MC system in the spinal cord might be involved in the increased pain state associated with the CCI lesion and prompted us to investigate whether tonic activity of the MC system contributed to this increased sensitivity. As shown in Figures 4–6, administration of SHU9119, an antagonist at the MC4 receptor, induced a significant anti-allodynic effect in both the cold and mechanical stimulation tests, indicated by an increased withdrawal latency upon immersion in a 4.5°C water bath and a higher threshold to von Frey stimulation, respectively. The observation that administration of an MC receptor antagonist produced hypoalgesia by itself indeed suggests a tonic influence of the MC system on nociceptive transmission.

The increase in MC receptor level in the superficial dorsal horn in CCI rats suggests an increased sensitivity for MC receptor agonists in neurons in this area. Treatment with the MC receptor agonist MTII resulted in an opposite effect compared with SHU9119, producing an increased sensitivity to both cold and mechanical stimulation. Similar results were obtained with D-Tyr-MTII, an MC receptor agonist that displays a higher affinity for the MC4 receptor compared with the MC3 receptor. Coadministration of MTII and SHU9119 demonstrated the specificity of the anti-allodynic effect of SHU9119. Injection of 15 ng of MTII, a dose which by itself caused no significant changes in nociceptive thresholds, completely blocked the anti-allodynic effect of 0.5 μg of SHU9119 when administered simultaneously, thereby demonstrating that the effects of these compounds are indeed mediated through the same receptor. Administration of the selective MC3 receptor agonist Nle-γ-MSH had no effect on sensitivity. Because both MTII and D-Tyr-MTII altered the responses to cold and von Frey stimulation whereas Nle-γ-MSH had no effect, we suggest that the observed changes in nociception are mediated through the MC4 receptor.

In the present study, we administered MC receptor ligands through a cannula placed in the cisterna magna, directly into the fluid surrounding the spinal cord. Adan et al. (1999) have demonstrated that a dose of 4.5 pmol of MTII is already sufficient to induce grooming when administered intracerebroventricularly. In contrast, we demonstrate that a >100-fold higher dose (500 ng, ~488 pmol) failed to induce grooming when injected into the cisterna magna. We cannot exclude the possibility that a small portion of the drugs administered into the cisterna magna will retrogradely reach the ventricular system and surrounding structures and that these structures play a role in the (anti-)nociceptive effects described here. However, because no grooming was ob-

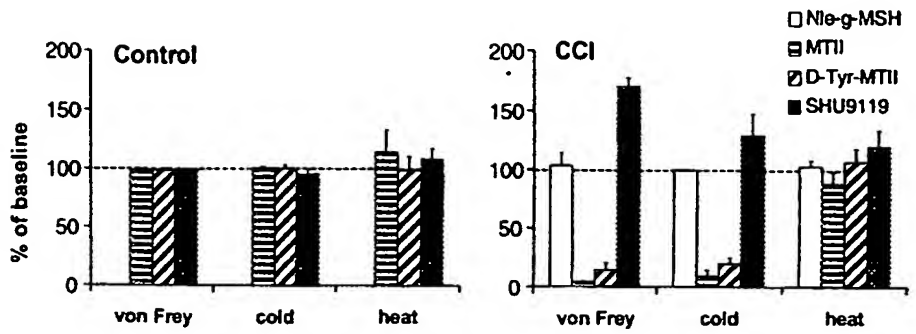


Figure 8. Summary of the effects of intrathecal administration of the MC3-R-selective ligand Nle-γ-MSH (5 μg), the MC4-R-selective ligands MTII (500 ng) and D-Tyr-MTII (3 μg), and the MC-R antagonist SHU9119 (1.5 μg) on the responses of CCI and control rats to different stimuli (indicated on *x*-axis). Values represent thresholds at 30 min (MTII, D-Tyr-MTII, and Nle-γ-MSH) or 15 min (SHU9119) after injection as a percentage of baseline threshold. Data are presented as mean ± SEM of 11 (control) or 10 (CCI) rats each, except 1.5 μg of SHU9119 (n = 4 CCI rats).

served after injection of a high dose of agonist, we suggest that the effects we observed in the present study are predominantly exerted at the spinal level.

In the dorsal horn, immunoreactivity has been demonstrated for the MC receptor agonists α-MSH and ACTH, as well as for the opioid peptide β-endorphin (Tsou et al., 1986), all of which are derived from the POMC peptide. Furthermore, the μ- and δ-opioid receptor subtypes, for which β-endorphin displays a high affinity, has also been demonstrated in the same area by immunocytochemistry (Chaplan et al., 1994; Zerari et al., 1994). Therefore, we hypothesize that the observed anti-allodynic effects of SHU9119 might be caused by blockade of a tonic influence of endogenous α-MSH on nociception through the MC4 receptor in the spinal cord. This could tip the balance in favor of the anti-nociceptive actions of β-endorphin, coreleased with α-MSH in the same POMC projection areas, thus producing analgesia.

The exact source of spinal POMC expression is not known; it might be intrinsic to the spinal cord (Plantinga et al., 1992; van der Kraan et al., 1999) but may also originate from a supraspinal source, namely the nucleus tractus solitarius (Tsou et al., 1986) or the hypothalamus (Cechetto and Saper, 1988; Elias et al., 1998). Elias et al. (1998) have demonstrated that hypothalamic POMC-expressing neurons innervate the interomedial lateral cell column (IML) at a thoracic level in which sympathetic preganglionic cells are located. In this region, MC4-R mRNA is also expressed (Mountjoy and Wild, 1998).

The melanocortin system is suggested to play a role in the regulation of autonomic function, because centrally administered melanocortins can increase sympathetic nerve activity (Dunbar and Lu, 2000), possibly through activation of the MC4 receptor (Mountjoy et al., 1994; Mountjoy and Wild, 1998; Dunbar and Lu, 1999). Although its exact role is still a matter of debate, there are several lines of research indicating that the sympathetic nervous system is involved in neuropathic pain (Price et al., 1989; Kim and Chung, 1991; Shir and Seltzer, 1991; Ringkamp et al., 1999). One of the potential mechanisms by which sympathetic activity influences nociception is through an increased norepinephrine responsiveness in C fibers, the primary afferents activated by noxious stimuli (for review, see Janig, 1985; Bennett, 1991; Janig et al., 1996).

In this present study, we only demonstrated changes in the MC system in areas of the spinal cord that correspond to sciatic nerve input. We cannot, however, exclude the possibility that changes also occur at other spinal levels or that the ligands we used act through MC receptors located more rostrally. Thus, an alternative explanation for the observed effects of MC receptor ligands on neuropathic pain might be a change in sympathetic activity, mediated at the level of the IML.

As shown in Figure 3, baseline values for von Frey and cold stimulation were significantly lower in CCI rats compared with control rats, confirming the development of mechanical and cold allodynia associated with the CCI lesion (Bennett and Xie, 1988; Attal et al., 1990). However, in contrast to other groups (Bennett and Xie, 1988; Attal et al., 1990; Kupers et al., 1992), we observe no differences in sensitivity to noxious heat between control rats and CCI rats. The reason for this discrepancy is not clear but may result from genetic variability between various rat strains. As suggested previously, this may lead to differences in predisposition for the development of neuropathic conditions (Wiesenfeld-Hallin et al., 1993) or in sensitivity to noxious stimuli because of variations in endogenous opiate systems or adrenergic sensitivity (Lee et al., 1997; Hoffmann et al., 1998).

Interestingly, we only observed effects of the MC receptor ligands in CCI rats and only in response to cold and mechanical stimulation. From a clinical point of view, this is promising because, in this study, the effects of melanocortins appear to be specific for the allodynia associated with a neuropathic pain state, without altering normal pain sensation by inducing a more general analgesia. This specificity for the hyperalgesia underlying neuropathic pain without affecting baseline pain detection has also been reported for the α2-adrenergic agonist tizanidine (Leiphart et al., 1995).

In summary, in this present study, we show that intrathecally administered MC receptor ligands alter the sensitivity to cold and mechanical stimulation in a rat model for neuropathic pain, the CCI. Our data suggest that these effects are mediated through the MC4 receptor located in the spinal cord. SHU9119 produces profound anti-allodynia, whereas MTII and D-Tyr-MTII increase sensitivity to cold and mechanical sensitivity. We therefore suggest that selective MC4 receptor antagonists may be of value in the treatment of neuropathic pain and that further research into the mechanisms through which the effects of these ligands are exerted is needed.

REFERENCES

Adan RA, Szklarczyk AW, Oosterom J, Brakkee JH, Nijenhuis WA, Schaaper WM, Meloen RH, Gispen WH (1999) Characterization of melanocortin receptor ligands on cloned brain melanocortin receptors and on grooming behavior in the rat. Eur J Pharmacol 378:249–258.

Attal N, Jazat F, Kayser V, Guilbaud G (1990) Further evidence for "pain-related" behaviours in a model of unilateral peripheral mononeuropathy. Pain 41:235–251.

Backonja M, Arndt G, Gombar KA, Check B, Zimmermann M (1994) Response of chronic neuropathic pain syndromes to ketamine: a preliminary study. Pain [Erratum (1994) 58:433] 56:51–57.

Basbaum AI, Gautron M, Jazat F, Mayes M, Guilbaud G (1991) The spectrum of fiber loss in a model of neuropathic pain in the rat: an electron microscopic study. Pain 47:359–367.

Bennett GJ (1991) The role of the sympathetic nervous system in painful peripheral neuropathy. Pain 45:221–223.

Bennett GJ, Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33:87–107.

Bertolini A, Poggioli R, Ferrari W (1979) ACTH-induced hyperalgesia in rats. Experientia 35:1216–1217.

Besse D, Lombard MC, Perrot S, Besson JM (1992) Regulation of opioid binding sites in the superficial dorsal horn of the rat spinal cord following loose ligation of the sciatic nerve: comparison with sciatic nerve section and lumbar dorsal rhizotomy. Neuroscience 50:921–933.

Carlton SM, Dougherty PM, Pover CM, Coggeshall RE (1991) Neuroma formation and numbers of axons in a rat model of experimental peripheral neuropathy. Neurosci Lett 131:88–92.

Cechetto DF, Saper CB (1988) Neurochemical organization of the hypothalamic projection to the spinal cord in the rat. J Comp Neurol 272:579–604.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53:55–63.

Chizh BA, Dickenson AH, Wnendt S (1999) The race to control pain: more participants, more targets. Trends Pharmacol Sci 20:354–357.
Choi Y, Raja SN, Moore LC, Tobin JR (1996) Neuropathic pain in rats is associated with altered nitric oxide synthase activity in neural tissue. J Neurol Sci 138:14–20.
Cone RD, Lu D, Koppula S, Vage DI, Klungland H, Boston B, Chen W, Orth DN, Pouton C, Kesterson RA (1996) The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. Recent Prog Horm Res 51:287–317.
Dunbar JC, Lu H (1999) Leptin-induced increase in sympathetic nervous and cardiovascular tone is mediated by proopiomelanocortin (POMC) products. Brain Res Bull 50:215–221.
Dunbar JC, Lu H (2000) Proopiomelanocortin (POMC) products in the central regulation of sympathetic and cardiovascular dynamics: studies on melanocortin and opioid interactions. Peptides 21:211–217.
Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK (1998) Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron 21:1375–1385.
Felsby S, Nielsen J, Arendt-Nielsen L, Jensen TS (1996) NMDA receptor blockade in chronic neuropathic pain: a comparison of ketamine and magnesium chloride. Pain 64:283–291.
Gispen WH, Wiegant VM, Greven HM, De Wied D (1975) The induction of excessive grooming in the rat by intraventricular application of peptides derived from ACTH: structure-activity studies. Life Sci 17:645–652.
Gispen WH, Buitelaar J, Wiegant VM, Terenius L, De Wied D (1976) Interaction between ACTH fragments, brain opiate receptors and morphine-induced analgesia. Eur J Pharmacol 39:393–397.
Glazer S, Portenoy RK (1991) Systemic local anesthetics in pain control. J Pain Symptom Manage 6:30–39.
Hama AT, Sagen J, Pappas GD (1994) Morphological characterization of dorsal horn spinal neurons in rats with unilateral constriction nerve injury: a preliminary study. Neurol Res 16:297–304.
Hoffmann O, Plesan A, Wiesenfeld-Hallin Z (1998) Genetic differences in morphine sensitivity, tolerance and withdrawal in rats. Brain Res 806:232–237.
Huang QH, Entwistle ML, Alvaro JD, Duman RS, Hruby VJ, Tatro JB (1997) Antipyretic role of endogenous melanocortins mediated by central melanocortin receptors during endotoxin-induced fever. J Neurosci 17:3343–3351.
Janig W (1985) Systemic and specific autonomic reactions in pain: efferent, afferent and endocrine components. Eur J Anaesthesiol 2:319–346.
Janig W, Levine JD, Michaelis M (1996) Interactions of sympathetic and primary afferent neurons following nerve injury and tissue trauma. Prog Brain Res 113:161–184.
Kajander KC, Xu J (1995) Quantitative evaluation of calcitonin gene-related peptide and substance P levels in rat spinal cord following peripheral nerve injury. Neurosci Lett 186:184–188.
Kim SH, Chung JM (1991) Sympathectomy alleviates mechanical allodynia in an experimental animal model for neuropathy in the rat. Neurosci Lett 134:131–134.
Kingery WS (1997) A critical review of controlled clinical trials for peripheral neuropathic pain and complex regional pain syndromes. Pain 73:123–139.
Kupers RC, Nuytten D, De Castro Costa M, Gybels JM (1992) A time course analysis of the changes in spontaneous and evoked behaviour in a rat model of neuropathic pain. Pain 50:101–111.
Lee DH, Chung K, Chung JM (1997) Strain differences in adrenergic sensitivity of neuropathic pain behaviors in an experimental rat model. NeuroReport 8:3453–3456.
Leiphart JW, Dills CV, Zikel OM, Kim DL, Levy RM (1995) A comparison of intrathecally administered narcotic and nonnarcotic analgesics for experimental chronic neuropathic pain. J Neurosurg 82:595–599.
Mao J, Price DD, Coghill RC, Mayer DJ, Hayes RL (1992) Spatial patterns of spinal cord [14C]-2-deoxyglucose metabolic activity in a rat model of painful peripheral mononeuropathy. Pain [Erratum (1992) 51:389] 50:89–100.
Mountjoy KG, Wild JM (1998) Melanocortin-4 receptor mRNA expression in the developing autonomic and central nervous systems. Brain Res Dev Brain Res 107:309–314.
Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD (1994) Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol 8:1298–1308.
Ollat H, Cesaro P (1995) Pharmacology of neuropathic pain. Clin Neuropharmacol 18:391–404.
Plantinga LC, Verhaagen J, Edwards PM, Schrama LH, Burbach JP, Gispen WH (1992) Expression of the pro-opiomelanocortin gene in dorsal root ganglia, spinal cord and sciatic nerve after sciatic nerve crush in the rat. Brain Res Mol Brain Res 16:135–142.
Price DD, Bennett GJ, Rafii A (1989) Psychophysical observations on patients with neuropathic pain relieved by a sympathetic block. Pain 36:273–288.
Ringkamp M, Eschenfelder S, Grethel EJ, Habler HJ, Meyer RA, Janig W, Raja SN (1999) Lumbar sympathectomy failed to reverse mechanical allodynia- and hyperalgesia-like behavior in rats with L5 spinal nerve injury. Pain 79:143–153.
Rosenberg JM, Harrell C, Ristic H, Werner RA, de Rosayro AM (1997) The effect of gabapentin on neuropathic pain. Clin J Pain 13:251–255.
Rowbotham MC, Reisner KL, Fields HL (1991) Both intravenous lidocaine and morphine reduce the pain of postherpetic neuralgia. Neurology 41:1024–1028.
Sandman CA, Kastin AJ (1981) Intraventricular administration of MSH induces hyperalgesia in rats. Peptides 2:231–233.
Schaaper WM, Adan RA, Posthuma TA, Oosterom J, Gispen WH, Meloen RH (1998) Synthesis of cyclic alpha-MSH peptides. Lett Pept Sci 5:205–208.
Shir Y, Seltzer Z (1991) Effects of sympathectomy in a model of causalgiform pain produced by partial sciatic nerve injury in rats. Pain 45:309–320.
Smock T, Fields HL (1981) ACTH1–24 blocks opiate-induced analgesia in the rat. Brain Res 212:202–206.
Stevens CW, Kajander KC, Bennett GJ, Seybold VS (1991) Bilateral and differential changes in spinal mu, delta and kappa opioid binding in rats with a painful, unilateral neuropathy. Pain 46:315–326.
Sugimoto T, Bennett GJ, Kajander KC (1990) Transsynaptic degeneration in the superficial dorsal horn after sciatic nerve injury: effects of a chronic constriction injury, transection, and strychnine. Pain 42:205–213.
Tatro JB (1993) Melanocortin receptors of the brain. In: Methods in neurosciences (Conn PM, ed), pp 87–104. New York: Academic.
Tatro JB (1996) Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides. Neuroimmunomodulation 3:259–284.
Tsou K, Khachaturian H, Akil H, Watson SJ (1986) Immunocytochemical localization of pro-opiomelanocortin-derived peptides in the adult rat spinal cord. Brain Res 378:28–35.
van der Kraan M, Tatro JB, Entwistle ML, Brakkee JH, Burbach JP, Adan RA, Gispen WH (1999) Expression of melanocortin receptors and pro-opiomelanocortin in the rat spinal cord in relation to neurotrophic effects of melanocortins. Brain Res Mol Brain Res 63:276–286.
Wiegant VM, Gispen WH, Terenius L, De Wied D (1977) ACTH-like peptides and morphine: interaction at the level of the CNS. Psychoneuroendocrinology 2:63–70.
Wiesenfeld-Hallin Z, Hao JX, Xu XJ, Aldskogius H, Seiger A (1993) Genetic factors influence the development of mechanical hypersensitivity, motor deficits and morphological damage after transient spinal cord ischemia in the rat. Pain [Erratum (1994) 57:135] 55:235–241.
Williams DWJ, Lipton JM, Giesecke AHJ (1986) Influence of centrally administered peptides on ear withdrawal from heat in the rabbit. Peptides 7:1095–1100.
Yaksh TL (1999) Spinal systems and pain processing: development of novel analgesic drugs with mechanistically defined models. Trends Pharmacol Sci 20:329–337.
Zerari F, Zouaoui D, Gastard M, Apartis E, Fischer J, Herbrecht F, Cupo A, Cucumel K, Conrath M (1994) Ultrastructural study of delta-opioid receptors in the dorsal horn of the rat spinal cord using monoclonal anti-idiotypic antibodies. J Chem Neuroanat 7:159–170.
Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16:109–110.

THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 304:818–826, 2003

VOL. 304, NO. 2
44826/1040611
Printed in U.S.A.

Anxiolytic-Like and Antidepressant-Like Activities of MCL0129 (1-[(S)-2-(4-Fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine), a Novel and Potent Nonpeptide Antagonist of the Melanocortin-4 Receptor

SHIGEYUKI CHAKI, SHIHO HIROTA, TAKEO FUNAKOSHI, YOSHIKO SUZUKI, SAYOKO SUETAKE, TAKETOSHI OKUBO, TAKAAKI ISHII, ATSURO NAKAZATO, and SHIGERU OKUYAMA

Medicinal Pharmacology Laboratory (S.C., S.H., T.F., Y.S., S.S.), Medicinal Chemistry Laboratory (T.O., T.I., A.N.), Medicinal Research Laboratories, and Ethical Business Strategy Division (S.O.), Taisho Pharmaceutical Co., Saitama, Japan

Received September 24, 2002; accepted October 28, 2002

ABSTRACT

We investigated the effects of a novel melanocortin-4 (MC4) receptor antagonist,1-[(S)-2-(4-fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine (MCL0129) on anxiety and depression in various rodent models. MCL0129 inhibited [^{125}I][Nle^4-D-Phe^7]-α-melanocyte-stimulating hormone (α-MSH) binding to MC4 receptor with a K_i value of 7.9 nM, without showing affinity for MC1 and MC3 receptors. MCL0129 at 1 μM had no apparent affinity for other receptors, transporters, and ion channels related to anxiety and depression except for a moderate affinity for the σ_1 receptor, serotonin transporter, and α_1-adrenoceptor, which means that MCL0129 is selective for the MC4 receptor. MCL0129 attenuated the α-MSH-increased cAMP formation in COS-1 cells expressing the MC4 receptor, whereas MCL0129 did not affect basal cAMP levels, thereby indicating that MCL0129 acts as an antagonist at the MC4 receptor. Swim stress markedly induced anxiogenic-like effects in both the light/dark exploration task in mice and the elevated plus-maze task in rats, and MCL0129 reversed the stress-induced anxiogenic-like effects. Under nonstress conditions, MCL0129 prolonged time spent in the light area in the light/dark exploration task and suppressed marble-burying behavior. MCL0129 shortened immobility time in the forced swim test and reduced the number of escape failures in inescapable shocks in the learned helplessness test, thus indicating an antidepressant potential. In contrast, MCL0129 had negligible effects on spontaneous locomotor activity, Rotarod performance, and hexobarbital-induced anesthesia. These observations indicate that MCL0129 is a potent and selective MC4 antagonist with anxiolytic- and antidepressant-like activities in various rodent models. MC4 receptor antagonists may prove effective for treating subjects with stress-related disorders such as depression and/or anxiety.

Stress initiates a complex cascade of responses that include endocrine, biochemical, and behavioral events. Many of these responses are initiated by release of corticotropin-releasing factor (CRF) (Owen and Nemeroff, 1991). In addition to activation of the brain CRF system, there are several lines of evidence that melanocortins (MCs), which stem from proopiomelanocortin by enzymatic processing, mediate important behavioral and biochemical responses to stress and, consequently, stress-induced disorders. Among MCs, it was reported that α-melanocyte-stimulating hormone (α-MSH) acts as a neurotransmitter or neuromodulator in the brain (Blasquez et al., 1991), and the relationship between α-MSH and adrenocorticotropic hormone (ACTH) and stress has been well documented. α-MSH and ACTH induce excessive grooming behavior in rats, a rodent behavioral response to stressful situations (De Barioglio et al., 1991; Adan et al., 1999), and antiserum to ACTH reduces novelty-induced grooming (Dunn et al., 1979). α-MSH and ACTH have been shown to inhibit the punish response in the Vogel conflict test in rats (Corda et al., 1990), and microinjection of α-MSH into the medial preoptic area and ventromedial nucleus increases anxiety and aggressive behavior (Gonzalez et al., 1996). Moreover, ACTH inhibits social contacts in the social interaction test in rats, an effect indicative of anxiogenic properties (File and Clarke, 1980), and increases isolation-induced distress vocalization in domestic chicks (Panksepp and Nor-

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.102.044826.

ABBREVIATIONS: CRF, corticotropin-releasing factor; MC, melanocortin; α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotropin; MCL0129, 1-[(S)-2-(4-fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine; NDP, [Nle^4-D-Phe^7]; PBS, phosphate-buffered saline; SET, serotonin transporter; SSRI, selective serotonin reuptake inhibitor; MCH, melanin-concentrating hormone.

mansell, 1990). ACTH also has been reported to activate the hypothalamic-pituitary-adrenal axis (Von Frijtag et al., 1998).

To date, five types of receptor subtype for MC (MC1 to MC5) have been reported. In the brain, mainly MC3 and MC4 are expressed, with little expression of MC5 (Gantz et al., 1993, 1994; Roselli-Rehfuss et al., 1993). The MC3 receptor is predominately located in the hypothalamus, whereas the MC4 receptor is ubiquitously distributed in the brain, including the limbic system (Gantz et al., 1993; Mountjoy et al., 1994). MC4 and MC5 receptors have been studied in knockout mice, and the former were shown to be involved in weight homeostasis (Huszar et al., 1997), whereas the MC5 receptor was found to have a role in functions of the exocrine glands (Chen et al., 1997). Among MC receptor subtypes, the MC4 receptor is of interest in terms of the relationship to stress and the regulation of emotional behavior, as based on the following findings. MC4 receptor agonists induce grooming behavior in rats, and the MC4 receptor antagonist, SHU9119, attenuates MC4 receptor agonist-induced grooming as well as novelty-induced grooming (Adan et al., 1999). The selective MC4 receptor antagonist, HS014, blocks immobilization stress-induced anorexia in rats (Vergoni et al., 1999), and the MC4 receptor was reported to be involved in activation of the hypothalamic-pituitary-adrenal axis (Von Frijtag et al., 1998); however, it was also found that MC4 receptor-selective antagonists did not elicit anxiolytic-like effects in the elevated plus-maze task in rats (Kask et al., 1998). Thus, the MC4 receptor might be involved in stress-induced changes in neurochemical and behavior-related responses. However, only recently have selective MC4 receptor antagonists been available, and the relationship between the MC4 receptor and stress-related behavior needs to be addressed using these pharmacological tools.

1-[(*S*)-2-(4-Fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine (MCL0129), a nonpeptide-selective MC4 receptor antagonist, was synthesized at our laboratories. We now report involvement of the MC4 receptor in stress-induced behavior such as depression and anxiety, using MCL0129 as a pharmacological tool.

Materials and Methods

Animals. Male ICR mice (20–30 g; Charles River, Yokohama, Japan) were housed 10 per cage. Male Sprague-Dawley rats (220–240 g; Charles River) were housed 3 per cage and used to assess stress-induced anxiogenic-like behavior in the elevated plus-maze task and antidepressant-like effects in the forced swimming test. For other behavioral studies, male Wistar rats (220–240 g; Charles River) were used. Animals were maintained under a 12-h light/dark cycle (light on at 7:00 AM) in a temperature- and humidity-controlled holding room. Food and water were available ad libitum. Behavioral studies were carried out between 9:00 AM and 4:00 PM. All studies were reviewed by the Taisho Pharmaceutical Co., Ltd. Animal Care Committee and met the Japanese Experimental Animal Research Association standards, as defined in the *Guidelines for Animal Experiments* (1987).

Chemicals. MCL0129 (Fig. 1) and fluvoxamine were synthesized in Taisho Pharmaceutical Laboratories. [^{125}I][Nle^4-D-Phe^7]α-Melanocyte-stimulating hormone (NDP-α-MSH) (specific radioactivity 81.4 TBq/mmol) and the cAMP assay system were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). COS-1 cells were purchased from American Type Culture Collection (Manassas, VA). α-MSH and NDP-α-MSH were purchased from Peninsula Laboratories (Belmont, CA). Diazepam and buspirone were purchased from Wako Chemicals (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO), respectively. All other chemicals used in this study were obtained commercially and were of the highest purity available. For the in vitro study, MCL0129 was dissolved in 0.1% dimethyl sulfoxide, and dimethyl sulfoxide (0.1%) did not affect binding assays and cAMP levels. For behavioral studies, MCL0129, diazepam, buspirone, and fluvoxamine were dissolved in 0.3% Tween 80/saline solution.

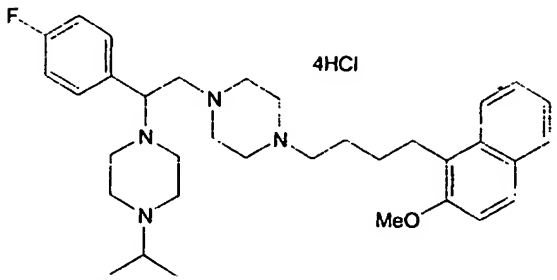


Fig. 1. Chemical structure of MCL0129

MC Receptor Expression Constructs, Cell Cultures, and Transfection. MC4, MC1, and MC3 receptor cDNAs were isolated using reverse transcriptase-polymerase chain reaction from the human hippocampus, WM-266–4 cells, and rat hypothalamus, respectively. MC4 and MC3 receptor cDNAs were cloned into expression vector pcDLΔPE and MC1 receptor cDNA into pTARGET. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37°C. The MC receptor cDNAs inserted into expression vectors were separately transfected into COS-1 cells using Lipofectin (Invitrogen, Carlsbad, CA), according to the protocol provided by the manufacturer (Felgner et al., 1987). At 72 h after transfection, COS-1 cells expressing MC1, MC3, or MC4 were used for pharmacological experiments.

[^{125}I]NDP-α-MSH Binding. COS-1 cells expressing the MC receptor were washed with PBS, scraped, and pelleted by centrifugation. Cell pellets were homogenized with 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA, 10 mM $CaCl_2$, and 100 μM phenylmethylsulfonyl fluoride, and centrifuged at 48,000*g* for 20 min at 4°C. The pellet was washed twice with the buffer, and the final pellet was suspended in assay buffer [50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA, 10 mM $CaCl_2$, 100 μM phenylmethylsulfonyl fluoride, and 0.1% bovine serum albumin] and used as the crude membrane preparation for binding studies. Protein concentration was determined according to the method reported by Bradford (1976). Binding assays of [^{125}I]NDP-α-MSH were done according to the method of Schioth et al. (1998). Membranes were incubated with [^{125}I]NDP-α-MSH (0.2 nM) for 120 min at 25°C. The reaction was terminated by rapid filtration over a GF/C filter presoaked with 0.5% bovine serum albumin, after which the filters were washed three times with the buffer. The radioactivity was quantified in a gamma-counter. Nonspecific binding was determined in the presence of 1 μM NDP-α-MSH. Specific binding was determined by subtracting nonspecific from total binding. In the competition assay, the concentration of the test compound that caused 50% inhibition of specific binding (IC_{50} value) was determined from each concentration-response curve. IC_{50} values were determined by the Marquardt-Levenberg nonlinear least-squares curve-fitting procedure, using the ORIGIN program (Origin LabCorp, Northampton, MA) running on Microsoft Windows 3.1.

Determination of cAMP. The effect of MCL0129 on cAMP formation was measured as we reported previously (Chaki et al., 1999), but with modification. COS-1 cells transiently expressing the MC4 receptor and grown in a six-well plate were used. The culture medium was removed, the cells were washed with PBS, and 1 ml of Dulbecco's modified Eagle's medium containing 1 mM isobutylmeth-

ylxanthine, a phosphodiesterase inhibitor, was added. The cells were incubated with α-MSH and/or MCL0129 for 15 min at 37°C; the culture medium was then aspirated and the cells were washed with PBS. Two milliliters of ice-cold 65% EtOH were added, and the cells were scraped from the wells. The supernatant was collected by centrifugation at 15,000 rpm for 15 min at 4°C. cAMP formed in the cells was determined, using a commercially available cAMP enzyme immunoassay system.

Stress-Induced Anxiogenic-Like Behavior in Mice. The swim stress consists of placing mice in a 20-cm-tall, 13-cm-wide cylindrical plastic container containing 10 cm of water maintained at 25 ± 1°C. Duration of the swim stress was 10 min, and the light/dark exploration test was done 10 min after the swim stress. The light/dark exploration test was carried out according to the method reported by Okuyama et al. (1999). The apparatus consisted of two polyvinylchloride boxes (20 × 20 × 14 cm) covered with Plexiglas; one of these boxes was darkened with cardboard. The light compartment was illuminated by a desk lamp (400 lux) placed 17 cm above the box, and the dark compartment provided the only room illumination. An opaque plastic tunnel (5 × 7 × 10 cm) separated the dark and the light compartments. During the observation, the experimenter always sat in the same place, next to the apparatus. The subjects were individually tested in 5-min sessions in the apparatus described above. Each mouse was placed in the center of the light area at the start of the test session. The amount of time spent in the light area was recorded for 5 min after the first entry into the dark area. A mouse whose four paws were in the next box was considered as having changed boxes. Mice were naive to the apparatus. MCL0129 was administered s.c. or p.o. 30 min before application of the swim stress. For nonstress control, vehicle was administered s.c. or p.o. 50 min before the test. Ten mice for vehicle and each for three dosages of compounds were used to generate dose-response reactions. When the effect of MCL0129 in nonstress conditions was investigated, the light/dark exploration test was run 30 min after the subcutaneous administration of MCL0129.

Marble-Burying Behavior in Mice. Marble-burying behavior was determined according to the method reported by Millan et al. (2000). Mice were individually placed in transparent, polycarbonate cages (22 × 32 × 13.5 cm) containing a 5-cm layer of sawdust and 24 glass marbles (1.5 cm in diameter) evenly spaced against the wall of the cage. Thirty minutes later, the animals were removed from the cages and the number of marbles at least two-thirds buried in the sawdust was recorded. The mice were treated s.c. 30 min before the test with either drug or vehicle.

Stress-Induced Anxiogenic-Like Behavior in Rats. The swim stress consists of placing rats in a 40-cm-tall, 20-cm-wide cylindrical plastic container containing 25 cm of water maintained at 25 ± 1°C. Duration of the swim stress was 2 min, and the elevated plus-maze test was done 5 min after the swim stress. The elevated plus-maze test was based on that validated for the rat by Guimaraes et al. (1991). The apparatus consisted of a plus-maze elevated 50 cm high from the floor. The apparatus consists of a plus-shaped maze elevated 50 cm from the floor and two opposite open arms, 50 × 10 cm, crossed at right angles by two arms of the same dimensions enclosed by 40 cm-high walls with an open roof. In addition, a 1-cm-high edge made of Plexiglas surrounded the open arms to avoid falls. Luminosity measured at the center of the maze was 80 lux. During the observation, the experimenter always sat in the same place, next to the apparatus. Each rat was placed in the center of the plus-maze facing one enclosed arm. The amount of time spent in open arms of the maze was recorded. Rats were naive to the apparatus. MCL0129 was administered p.o. 30 min before the swim stress.

Learned Helplessness Test in Rats. The learned helplessness test, using the shuttle box test, was carried out according to the method reported by Takamori et al. (2001a) as a model of behavioral despair. The two-way shuttle box (56 × 21 × 25 cm; Muromachi-Kikai, Tokyo, Japan) was divided into equal-sized chambers with use of a steel divider. Floors of the chambers in the shuttle box consisted of stainless steel rods. Scrambled shocks were delivered through a shock generator (SGS-001; Muromachi-Kikai). Rats were given MCL0129 s.c. 60 min before the inescapable shocks, and on day 1, the rats were individually placed in the chamber and given 90 inescapable shocks (1.8 mA) of 10 s duration at 2-s intervals. Control rats were not given shocks. On day 2, the rats were subjected to the 40-trial escape test. The animals were individually placed in the shuttle box and given a 5-min adaptation period; a tone signal was given during the first 5 s of each trial. If there was no avoidance response within this period, the tone signal remained on and a 1.8-mA shock (10-s duration) was delivered through the grid floor. If no escape response was made within this period, both the tone signal and the shock were automatically terminated. The intertrial interval was 5 s. The number of escape failures, which refers to a noncrossing response during the shock delivery, was recorded.

Forced Swimming Test in Rats. The effect of the compound was evaluated by both the method described by Porsolt (1978) and by a time-sampling technique. A time-sampling technique was used to score several types of behavior (immobility, swimming, climbing) as described by Detke et al. (1995). The swimming sessions were carried out according to the method described by Detke et al. (1995) and was similar to that described by Porsolt et al. (1978), except that the water was deeper. Swimming sessions were conducted by placing rats in cylinders containing 25°C water, 30 cm deep, so that rats could not support themselves by touching the bottom with their feet. Two swimming sessions were conducted between 10:00 AM and 4:00 PM: an initial 15-min pretest followed 24 h later by a 5-min test. MCL0129 was administered s.c. during the period between these two sessions (24 and 0.5 h before the test). Following both swimming sessions, the rats were removed from the cylinders, placed in a heated cage for 15 min, and then returned to their home cages. Test sessions were videotaped from the front of the cylinders for later scoring. The water in the cylinders was changed after every trial. A time-sampling technique was used to score behavior during a single viewing. This method has previously been described by Detke et al. (1995) and was shown to be reliable and valid for detecting effects of different antidepressant drugs. At the end of each 5-s period during the test session, the scorer rated the rat's behavior as one of the following three behaviors: 1) immobility, floating in the water without struggling, and making only movements necessary to keep its head above water; 2) swimming, making active swimming motions between quadrants of the cylinder, more than necessary to merely keep the head above water, moving around in the cylinder; and 3) climbing movements with forepaws in and out of the water, usually directed against the walls.

Spontaneous Locomotor Activity in Rats. Spontaneous locomotor activity was determined as reported (Okuyama et al., 1999). Animals were housed individually in transparent acrylic cages (47 × 28.5 × 29.5 cm), and spontaneous locomotor activity was recorded every 10 min for 60 min, using a SCANET apparatus (Neuroscience Inc., Tokyo, Japan) placed in a sound-proof box. MCL0129 was administered s.c. 30 min before the start of measurements.

Rotarod Performance in Rats. Rotarod performance was carried out as reported previously (Okuyama et al., 1999). The Rotarod (Campden Instruments, Leicestershire, UK), consisted of a gritted plastic roller (3 cm in diameter, 9 cm long) flanked by two large round plates to prevent the animal from escaping, and was run at 10 rpm. All animals were given control trials before the test. A rat was placed on the roller, and the length of time it remained on the rod was measured. A maximum of 2 min was allowed for each animal. MCL0129 was administered s.c. 30 min before the test.

Potentiation of Hexobarbital-Induced Anesthesia in Rats. Hexobarbital-induced anesthesia was given as described (Okuyama et al., 1999). Hexobarbital-induced anesthesia was estimated based on duration of the righting reflex loss. Hexobarbital (100 mg/kg i.p.) was administered 30 min after the subcutaneous administration of MCL0129.

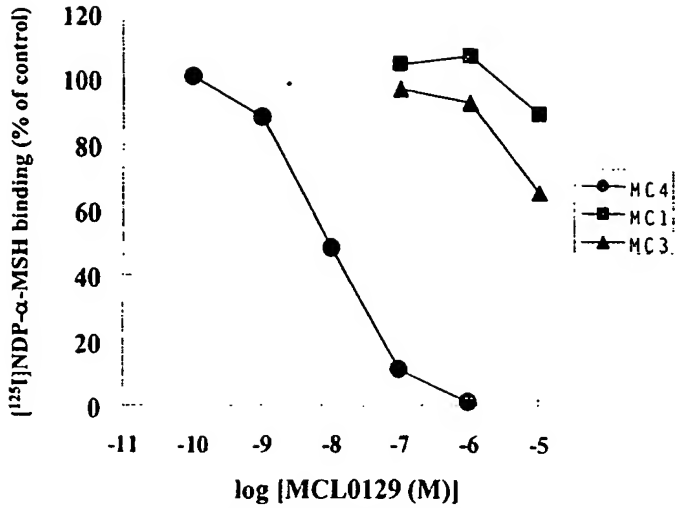


Fig. 2. Inhibition of NDP-α-MSH binding to recombinant MC4 (●), MC1 (■), and MC3 (▲) by MCL0129. Receptor binding assay of MC receptors was done as described under *Materials and Methods*. Results are the mean value of three separate experiments, each done in duplicate.

Statistical Analysis. Data from in vivo experiments were analyzed by one-way analysis of variance, and significant differences between groups were determined using Dunnett's test. In the case of the learned helplessness test, a comparison between two groups was made using the Mann-Whitney *U* test. Between-group comparisons were assessed using the Steel test.

Results

In Vitro Receptor Profiles of MCL0129. MCL0129 inhibited [^{125}I]NDP-α-MSH binding to membranes of COS-1 cells expressing the human MC4 receptor with a K_i value of 7.9 ± 0.35 nM (Fig. 2). By contrast, MCL0129 showed no affinity for the human MC1 receptor and the rat MC3 receptor, even at 10 μM, when assessed by [^{125}I]NDP-α-MSH to membranes of COS-1 cells expressing each receptor (Fig. 2). MCL0129 dose dependently inhibited the cAMP formation induced by α-melanin-concentrating hormone (α-MCH) in COS-1 cells transiently expressing the MC4 receptor, without affecting the basal cAMP level per se (Fig. 3), thereby indicating that MCL0129 acts as an antagonist at the MC4 receptor. MCL0129 showed moderate affinity for the σ1 receptor (IC_{50} = 68.9 nM), the serotonin transporter (SET) (IC_{50} = 383 nM) and the α_1-adrenoceptor (IC_{50} = 630 nM) (Table 1). MCL0129 did not show affinity for other receptors and transporters, including σ2, CRF1, opiates δ and μ, opioid receptor-like-1 (ORL-1), D_2, *N*-methyl-D-aspartate receptors, α_{2A}- and α_{2C}-adrenoceptors, norepinephrine transporter, glutamate transporter, and the Ca^{2+} channel even at 1 μM (Table 1).

Effect on Stress-Induced Anxiogenic-Like Behavior in Mice and Rats. We reported that time spent in the light area in the light/dark exploration task (mice) and time spent on the open arms in the elevated plus-maze task (rats) was significantly reduced by swim stress, and the anxiogenic-like behavior was attenuated by CRF1 receptor antagonists as did diazepam (Okuyama et al., 1999). In the present study, swim stress significantly [$F(1,18) = 40.2$, $p < 0.01$ for s.c.; $F(1,18) = 10.8$, $p < 0.01$ for p.o.] reduced the time in the light area in mice, and MCL0129 significantly and dose dependently attenuated the decrease in time spent in the light area when administered either orally [$F(3,36) = 2.95$, $p < 0.05$] or subcutaneously [$F(3,36) = 3.60$, $p < 0.05$] (Fig. 4). Likewise, the 2-min swim stress significantly [$F(1,28) = 32.8$, $p < 0.01$] decreased the time spent on the open arms in the elevated plus-maze task in rats. MCL0129, when administered p.o., dose dependently and significantly [$F(4,70) = 7.73$, $p < 0.01$] (10 and 30 mg/kg) ameliorated anxiogenic-like behavior caused by swim stress (Fig. 5). Practically the same result was obtained when assessed by the ratio of open arm entries/total arm entries (data not shown).

Effect of MCL0129 in the Light/Dark Exploration Test in Naive Mice. MCL0129 prolonged the time spent in the light area in the light/dark exploration task in mice in a

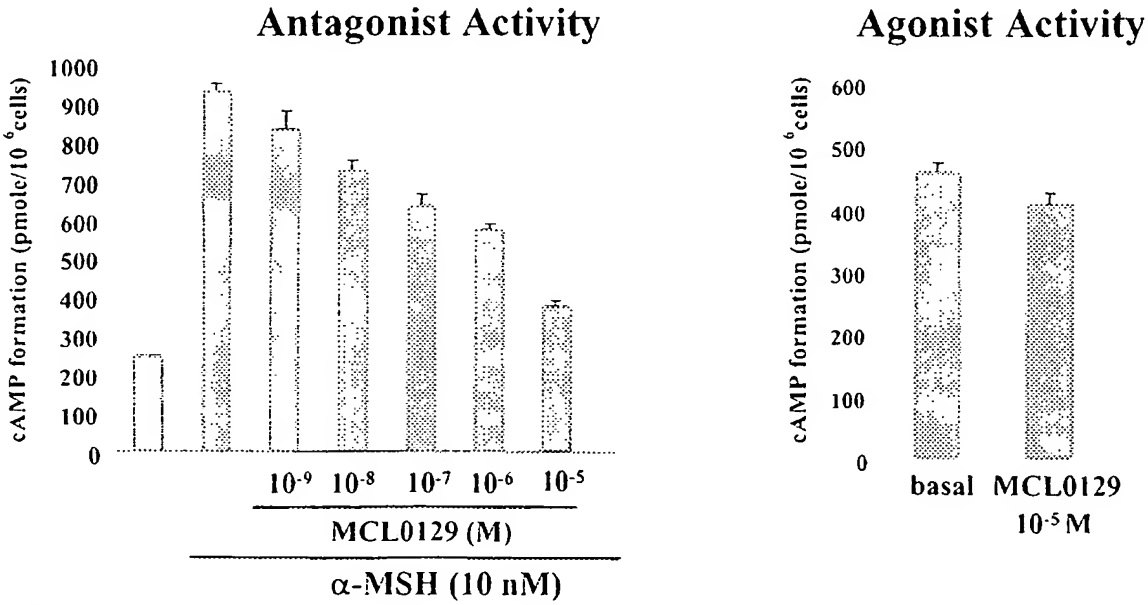


Fig. 3. Effect of MCL0129 on basal and α-MSH-induced increase in cAMP accumulation in COS-1 cells expressing the MC4 receptor. MCL0129 was incubated with or without 10 nM α-MSH for 15 min; then, cAMP formed in the cells was measured as described under *Materials and Methods*. Results are mean ± S.E. obtained from three experiments.

TABLE 1

Receptor selectivity of MCL0129

Effect on EAAT2 was evaluated by [^{3}H]glutamate uptake by Y79 cells, and effects on other receptors, transporters and channels were evaluated by receptor binding.

Receptors/ Transporters	Source	Radioligand	IC50
			nM
SET	Rat cortex	[^{3}H]Paroxetine	383
NET	Rat cortex	[^{3}H]Nisoxetine	>1000
α_1	Rat cortex	[^{3}H]MK912	630
α_{2A}	Human recombinant	[^{3}H]MK912	>1000
α_{2C}	Human recombinant	[^{3}H]MK912	1000
D2	Rat striatum	[^{3}H]Raclopride	>1000
σ_1	Guinea pig brain	[^{3}H](+)-Pentazocine	68.9
σ_2	Guinea pig brain	[^{3}H]DTG	>1000
CRF1	Monkey amygdala	^{125}I-Ovine CRF	>1000
opiate δ	Rat brain	[^{3}H]DPDPE	>1000
opiate μ	Rat brain	[^{3}H]DAMGO	>1000
ORL1	Human recombinant	[^{125}I]Nociceptine	>1000
NMDA	Rat brain	[^{3}H]CGP39653	>1000
EAAT2	Y79 cells	[^{3}H]Glutamate	>1000
Ca^{2+} channel	Rat brain	[^{3}H](+)-PN200-110	>1000

NET, norepinephrine transporter; [^{3}H]DTG, 1,3-di-*o*-tolyguanidine; [^{3}H]DPDPE, [D-Pen^2, D-Pen^5]-enkephalin; [^{3}H]DAMGO, [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin; ORL1, opioid receptor-like-1; NMDA, *N*-methyl-D-aspartate; EAAT2, excitatory amino acid transporter 2.

dose-dependent and significant manner [$F(3,36) = 3.44$, $p < 0.05$] (Fig. 6).

Effect of MCL0129 in Marble-Burying Behavior in Mice. Anxiolytics such as diazepam [$F(3,36) = 8.24$, $p < 0.01$] and buspirone [$F(3,36) = 15.8$, $p < 0.01$] significantly reduced the marble-burying behavior (Fig. 7c and d). Likewise, fluvoxamine, a selective serotonin reuptake inhibitor (SSRI), potently and significantly blocked this activity [$F(3,36) = 9.02$, $p < 0.01$] (Fig. 7b). MCL0129, at 10 mg/kg, also significantly reduced the marble-burying behavior [$F(3,36) = 5.2$, $p < 0.01$] (Fig. 7a).

Effect of MCL0129 in Forced Swimming Test in Rats. MCL0129 at 10 and 30 mg/kg s.c. significantly and dose dependently reduced the immobility time [$F(3,36) = 6.98$, $p < 0.01$] (Fig. 8a). When assessed according to the behavioral scoring method reported by Detke et al. (1995), MCL0129 selectively and significantly increased the swimming behavior without affecting the climbing behavior (Fig. 8b).

Effect of MCL0129 in the Learned Helplessness Test in Rats. As compared with control animals, nonstressed animals exposed to inescapable shock (helpless) manifested a significantly higher number of escape failures ($p < 0.01$). Acute administration of MCL0129, when administered before an inescapable shock (acquisition phase), dose dependently and at 3 mg/kg ($p < 0.01$) and 10 mg/kg ($p < 0.05$) s.c. significantly decreased the number of escape failures (Fig. 9).

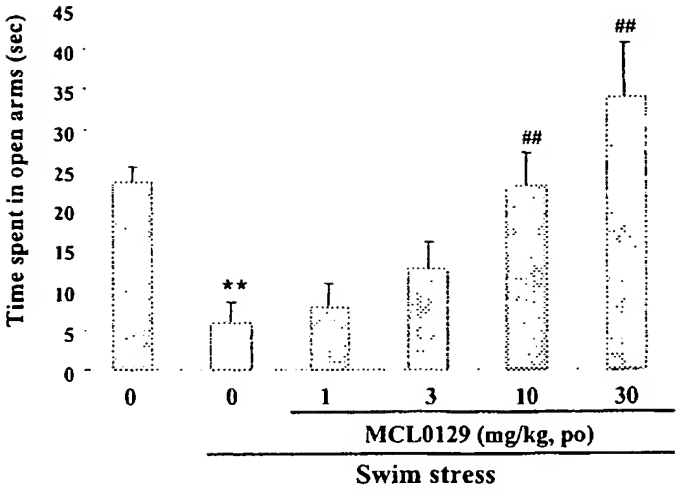


Fig. 5. Effect of MCL0129 on swim stress-induced reduction of time spent in open arms in the elevated plus-maze task in rats. Data represent mean ± S.E. ($n = 15$). **, $p < 0.01$ versus nonstress group (Dunnett's test); ##, $p < 0.01$ versus swim stress group (Dunnett's test).

Effect of MCL0129 on General Behavior. MCL0129 significantly inhibited spontaneous locomotor activity at a relatively higher dose of 100 mg/kg s.c. [$F(3,28) = 6.86$, $p < 0.01$], compared with pharmacologically effective dosages (Fig. 10a). MCL0129 did not affect Rotarod performance [$F(3,36) = 0.63$, $p = 0.76$;0.26, n.s.] and hexobarbital-induced sleeping time [$F(3,36) = 1.16$, $p = 0.63$;0.24, n.s.] (1, 10, and 100 mg/kg s.c.), whereas diazepam potently and significantly affected these functions in the previous study (Okuyama et al., 1999) (Fig. 10, b and c).

Discussion

We obtained evidence that MCL0129 is a potent and selective MC4 receptor antagonist and that MCL0129 showed

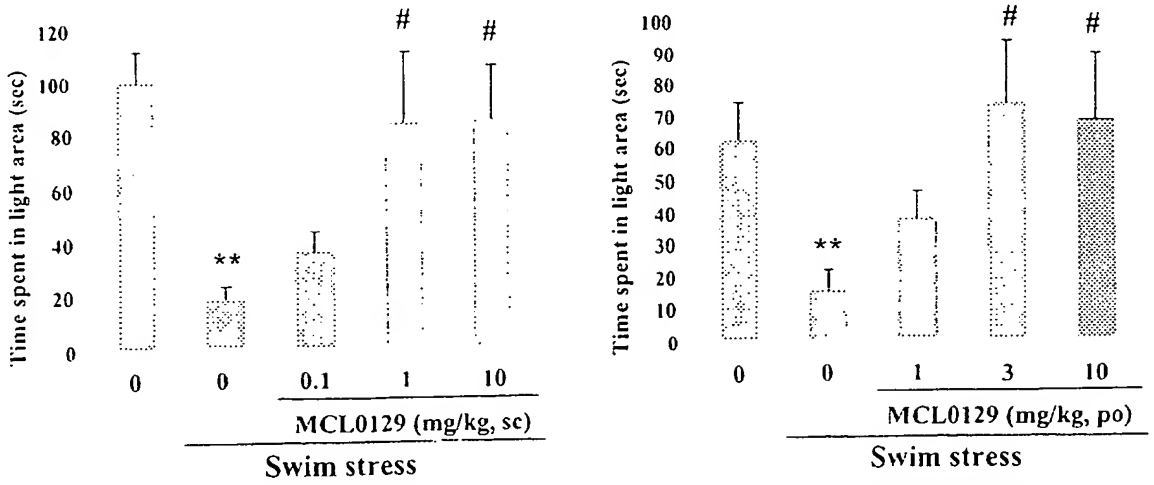


Fig. 4. Effect of MCL0129 on swim stress-induced reduction of time spent in the light area in the light/dark exploration test in mice. MCL0129 was administered either orally or subcutaneously. Data represent mean ± S.E. ($n = 10$). **, $p < 0.01$ versus nonstress group (Dunnett's test); #, $p < 0.05$ versus swim stress group (Dunnett's test).

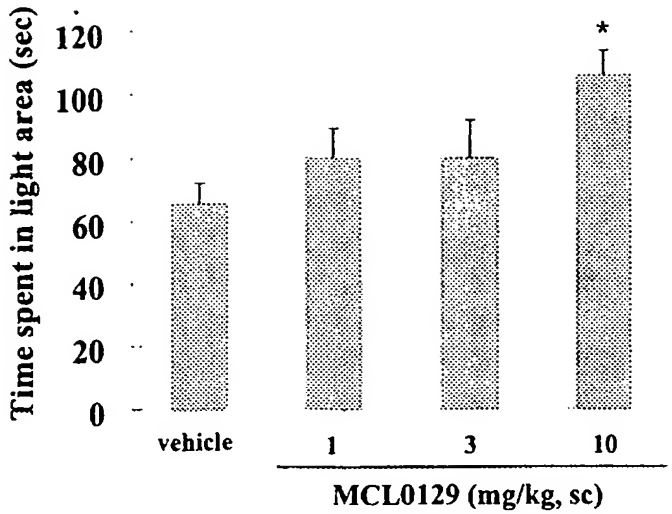


Fig. 6. Effect of MCL0129 on time spent in the light area in the light/dark exploration test in mice (nonstress condition). Data represent mean ± S.E. (n = 10). *, $p < 0.05$ versus vehicle-treated group (Dunnett's test).

antidepressant-like and anxiolytic-like activities in various rodent models.

MCL0129 inhibited [^{125}I]NDP-α-MSH binding to the recombinant human MC4 receptor without affecting [^{125}I]NDP-α-MSH binding to other MC receptor subtypes such as MC1 and MC3. By contrast, MCL0129 showed moderate to negligible affinities for other stress- and anxiety/depression-related receptors and transporters. Moreover, MCL0129 attenuated the α-MSH-induced increase in cAMP formation in COS-1 cells expressing the MC4 receptor, whereas MCL0129 itself had no effect on basal levels of cAMP. These in vitro data clearly show that MCL0129 is a selective MC4 receptor antagonist. Because MCL0129 is the most potent and selective nonpeptide MC4 receptor antagonist reported hitherto and is highly selective among MC receptors, MCL0129 should prove to be a useful pharmacological tool for investigation of physiological roles of MC4 receptor.

Stress-induced anxiogenic-like behavior has been used as a model of anxiety, and the swim stress markedly reduced the time spent in the light area in light/dark exploration tests in mice and in open arms in the elevated plus-maze task in rats, both of which were ameliorated previously by the administration of diazepam as well as CRF1 receptor antagonists (Okuyama et al., 1999). In the present study, MCL0129 dose dependently and significantly attenuated the swim stress-induced anxiogenic-like effect in both paradigms. Moreover, MCL0129 significantly prolonged the time spent in the light area in light/dark exploration tests on naive mice. In a previous study, CRF1 receptor antagonists did not show significant effects in this model (Okuyama et al., 1999). Therefore, MC4 receptor antagonists may have different pharmacological profiles from those of CRF1 receptor antagonists in terms of anxiolytic-like activity.

SSRIs, as well as benzodiazepine anxiolytics, have been reported to suppress marble burying without disrupting general behavior (Millan et al., 2001). Although it remains to be established whether blockade of marble-burying behavior in

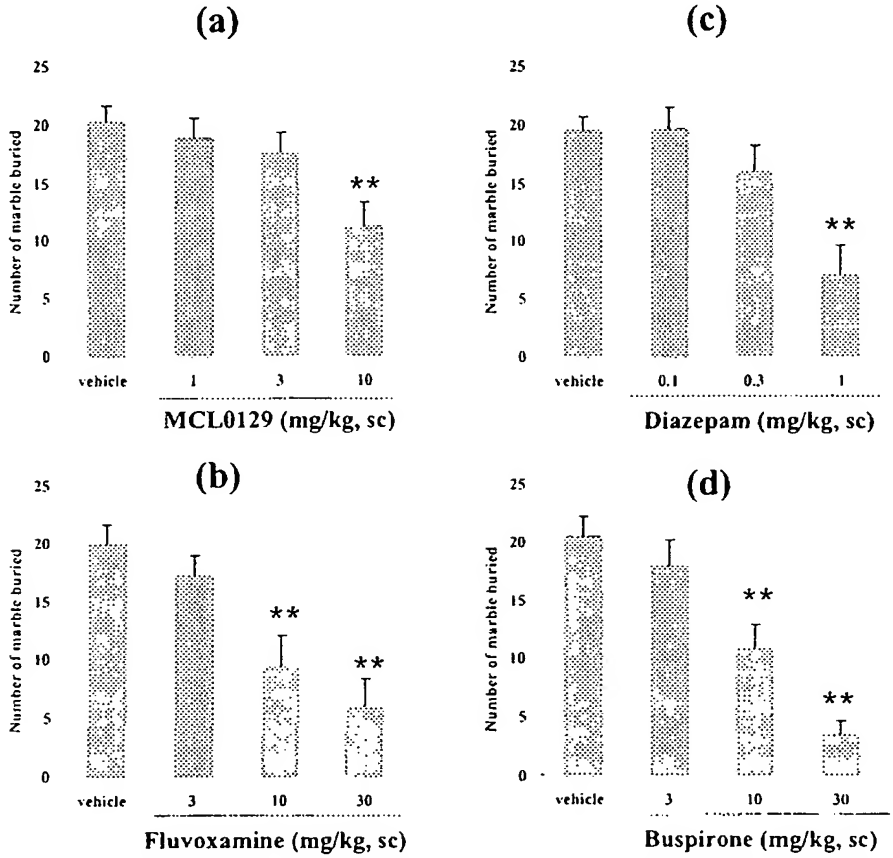


Fig. 7. Effect of MCL0129 (a), fluvoxamine (b), diazepam (c), and buspirone (d) on marble-burying behavior in mice. Data represent mean ± S.E. (n = 10). **, $p < 0.01$ versus vehicle-treated group (Dunnett's test).

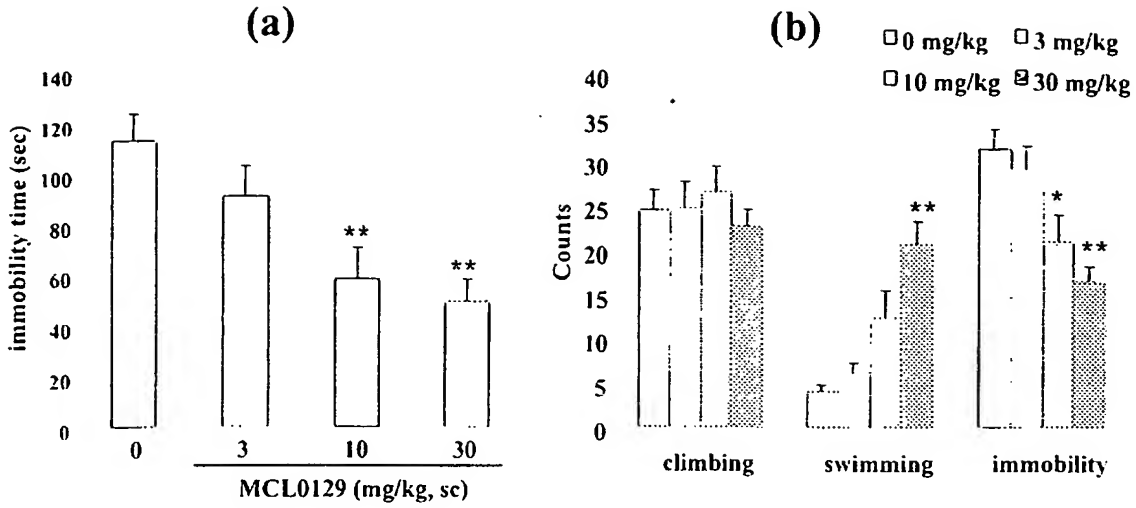


Fig. 8. Effect of MCL0129 in forced swimming tests in rats. The effect of MCL0129 was evaluated by both the method (a) described by Porsolt et al. (1978) and the time-sampling technique (b) described by Detke et al. (1995). Data represent mean ± S.E. (n = 10). *, $p < 0.05$, **, $p < 0.01$ versus vehicle-treated group (Dunnett's test).

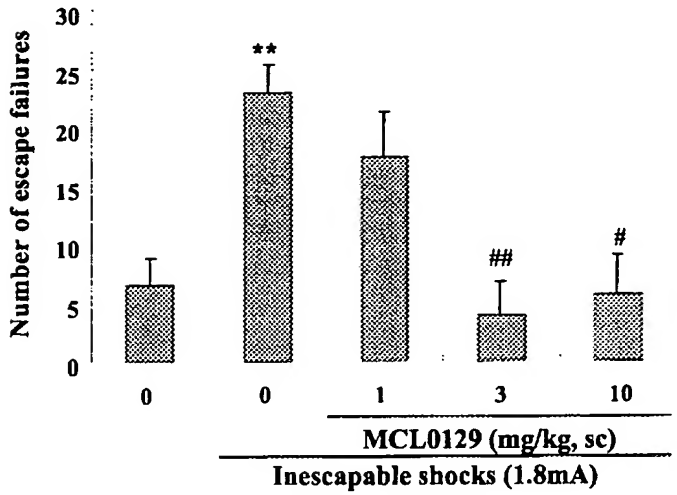


Fig. 9. Effect of MCL0129 in the learned helplessness test in rats. Data represent mean ± S.E. (n = 10). **, $p < 0.01$ versus noninescapable shock group (Mann-Whitney U test). #, $p < 0.05$, ##, $p < 0.01$ versus inescapable shock group (Steel test).

mice is predictive of clinically relevant anti-impulsive properties, this action is of interest in view of the increasing utility of SSRIs in the treatment of subjects with obsessive-compulsive disorders (Pigott and Seay, 1999). In this study, anxiolytics such as diazepam and buspirone as well as the SSRI, fluvoxamine, blocked marble-burying behavior, as reported previously (Millan et al., 2001). In the marble-burying test, MCL0129 was effective in significantly reducing this compulsive burying of novel objects.

MCL0129 significantly shortened immobility time in forced swimming tests in rats. When assessed by a behavioral sampling method, MCL0129 increased swimming behavior without affecting climbing behavior. In this test, SSRIs such as fluvoxamine and fluoxetine specifically increased the swimming score, whereas noradrenaline reuptake inhibitors such as reboxetine increased climbing behavior without affecting swimming (Detke et al., 1995, 1996; Cryan et al., 2002). Thus, it is presumed that compounds that potentiate serotonin transmission may increase the swimming score, whereas drugs with actions on norepinephrine transmission may increase climbing scores. In light of this hypothesis, it is likely that MCL0129 exerted antidepressant-like activity in the forced swimming test by acting on serotonin transmission. This result is consistent with the observation regarding marble-burying behavior in which both MCL0129 and SSRIs were effective. Whether or not the MC4 receptor antagonist influences serotonin transmission remains to be investigated.

We further evaluated the antidepressant potential of the MC4 receptor antagonist in the learned helplessness test in rats. Under the same conditions used in this test, we reported that fluvoxamine and imipramine showed antidepressant-like effects only when administered subchronically for 8 days, whereas CRF1 receptor antagonists showed activity even in cases of acute administration (Takamori et al., 2001a,b). MCL0129, when administered under the same schedule as those compounds, in acquisition phase rather than in consolidation and retention phases, exhibited antidepressant-like effects even with an acute administration. Therefore, MC4 receptor antagonists, like CRF1 receptor antagonists, may exert antidepressant-like activity with an early onset. It was reported that the MC4 receptor is involved in pain, and MC4 receptor agonists increased the sensitivity to mechanical and cold stimulation, whereas MC4 receptor antagonists alleviated cold and mechanical allodynia in a rat model of neuropathic pain (Vrinten et al., 2000, 2001). Therefore, involvement of decrease in pain threshold in this test by MC4 receptor agonists needs to be ruled out.

Many drugs that act on the central nervous system often cause unwanted side effects such as prolongation of sleeping, sleep sedation (Okuyama et al., 1999), and impaired motor coordination (Bristow et al., 1996). In the present study, MCL0129 did not affect hexobarbital-induced sleeping time and Rotarod performance in rats, even at the highest doses given. MCL0129 inhibited spontaneous locomotor activity, but this effect occurred at the highest doses of 100 mg/kg MCL0129. Therefore, MC4 receptor antagonists are expected to be without the unwanted central nervous system side effects sometimes seen in patients on antidepressants and/or anxiolytics, although concern for body weight gain with

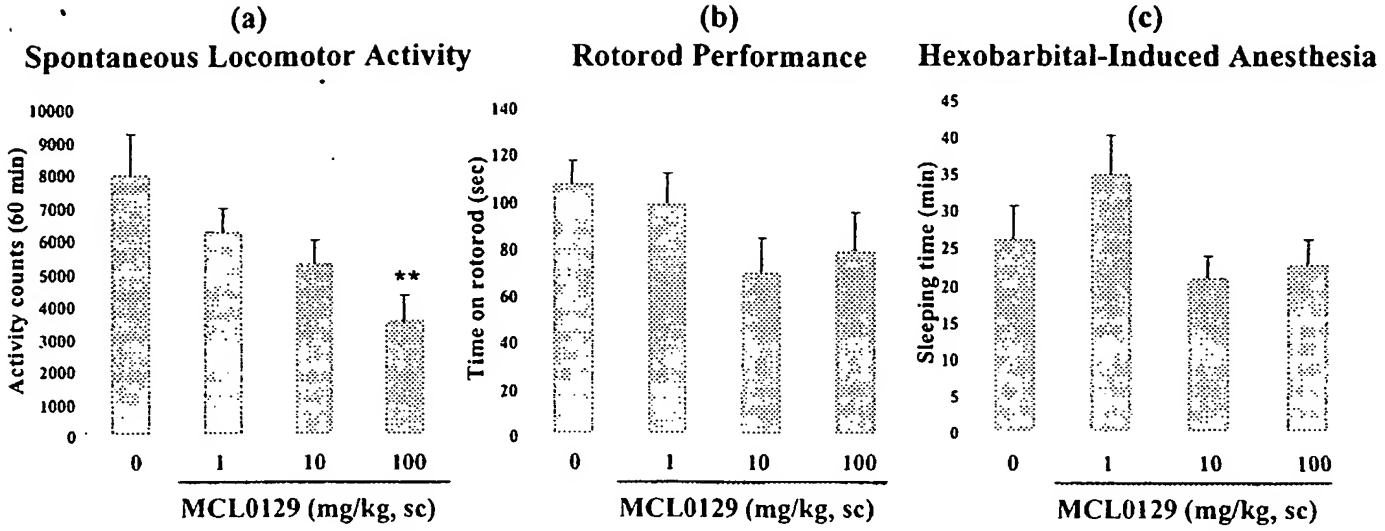


Fig. 10. General behavioral profiles of MCL0129: effect on spontaneous locomotor activity (a), Rotarod performance (b), and hexobarbital-induced anesthesia (c). Data represent mean ± S.E. (n = 6 for locomotor activity, n = 8 for Rotarod and hexobarbital-induced anesthesia). **, $p < 0.01$ versus vehicle-treated group (Dunnett's test).

chronic administration remains, as MC4 receptor antagonism was reported to lead to obesity (Huszar et al., 1997).

MCL0129 showed moderate affinity for SET in receptor binding studies. With regard to possible involvement of blockade of SET in the pharmacological actions of MCL0129, fluvoxamine, a SSRI, did not show effects on swim stress-induced anxiogenic-like behavior in rats or mice and did not show anxiolytic-like activity in the light/dark exploration test in naive mice (unpublished data). Moreover, fluvoxamine did not have antidepressant activity in the learned helplessness test in the case of an acute administration, whereas it was effective in repeated administration in the present experimental condition (Takamori et al., 2001b). These results clearly show that the effects of MCL0129 are mediated through the MC4 receptor, although involvement of the σ1 receptor needs to be ruled out.

There are reports which suggest that the brain melanocortinergic system might be involved in stress-related behaviors (Corda et al., 1990; de Barioglio et al., 1991; Gonzalez et al., 1996; Adan et al., 1999), and blockade of the MC receptor may lead to anxiolytic-like activity (Dunn et al., 1979; Adan et al., 1999; Vergoni et al., 1999). In a preliminary study, we observed that the peptide MC4 receptor agonist, Ac-[NIle^4,Asp^5,D-Phe^7,Lys^{10}]-α-MSH-4-10-NH_2, showed anxiogenic-like effects in the Vogel test on rats, and peptidomimetic MC4 receptor antagonists exhibited anti-stress activities in both rats and mice, which means that the MC4 receptor may be involved in stress-related behavior (S. Chaki, S. Ogawa, Y. Toda, T. Funakoshi, and S. Okoyama, unpublished data). These observations are consistent with the present results that MC4 receptor-selective antagonist had antidepressant-like and anxiolytic-like activity in various rodent models. Recently, it was reported that MCH receptor antagonist showed anxiolytic-like and antidepressant-like activities as well as anorectic activity in rodents (Borowsky et al., 2002). It is interesting that MCH and MC act differently in food intake, yet show the same effects in emotional behaviors.

In conclusion, the brain melanocortin system may be involved in stress-related disorders such as anxiety and depression, and MC4 receptor blockade may be a useful approach to treat subjects with anxiety and depression, and without the side effects sometimes seen with medication with anxiolytics and antidepressants. Moreover, MCL0129 is a useful pharmacological tool to investigate involvement of the MC4 receptor in such disorders and to elucidate mechanisms mediated through the MC4 receptor.

References

Adan RA, Szklarczyk AW, Oosterom J, Brakkee JH, Nijenhuis WA, Schaaper WM, Meloen RH, and Gispen WH (1999) Characterization of melanocortin receptor ligands on cloned brain melanocortin receptors and on grooming behavior in the rat. *Eur J Pharmacol* 378:249–258.

Blasquez C, Jegou S, Bunel DT, Delbende C, Braquet P, and Vaudry H (1991) Central-type benzodiazepines inhibit release of α-melanocyte-stimulating hormone from the rat hypothalamus. *Neuroscience* 42:509–516.

Borowsky B, Durkin MM, Ogozalek K, Marzabadi MR, Deleon J, Heurich R, Lichtblau H, Shaposhnik Z, Daniewska I, Blackburn TP, et al. (2002) Antidepressant, anxiolytic and anorectic effects of a melanin-concentrating hormone-1 receptor antagonist. *Nature Med* 8:825–830.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.

Bristow LJ, Flatman KL, Hutson PH, Kulagowski JJ, Leeson PD, Young L, and Tricklebank MD (1996) The atypical neuroleptic profile of the glycine/N-methyl-D-aspartate receptor antagonist, L-701,324, in rodents. *J Pharmacol Exp Ther* 277:578–585.

Chaki S, Okuyama S, Nakazato A, Kumagai T, Okubo T, Ikeda Y, Oshida Y, Hamajima Y, and Tomisawa K (1999) In vitro pharmacological profile of nonpeptide CRF1 receptor antagonists, CRA1000 and CRA1001. *Eur J Pharmacol* 371: 205–211.

Chen W, Kelly MA, Opitz-Araya X, Thomas RE, Low MJ, and Cone RD (1997) Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell* 91:789–798.

Corda MG, Orlandi M, and Fratta W (1990) Proconflict effect of ACTH 1–24: interaction with benzodiazepines. *Pharmacol Biochem Behav* 36:631–634.

Cryan JF, Page ME, and Lucki I (2002) Noradrenergic lesions differentially alter the antidepressant-like effects of reboxetine in a modified forced swim test. *Eur J Pharmacol* 436:197–205.

De Barioglio SR, Lezcano N, and Celis ME (1991) Alpha MSH-induced excessive grooming behavior involves a GABAergic mechanism. *Peptides* 12:203–205.

Detke MJ and Lucki I (1996) Detection of serotonergic and noradrenergic antidepressants in the rat forced swimming test: the effects of water depth. *Behav Brain Res* 73:43–46.

Detke MJ, Rickels M, and Lucki I (1995) Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology* 121:66–72.

Dunn AJ, Green EJ, and Isaacson RL (1979) Intracerebral adrenocorticotropic hormone mediates novelty-induced grooming in the rat. *Science (Wash DC)* 203:281–283.

Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz H, Northrop JP, Ringold GM, and Danielsen M (1987) Lipofectin: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 84:7413–7417.

File SE and Clarke A (1980) Intraventricular ACTH reduces social interaction in male rats. *Pharmacol Biochem Behav* 12:711–715.

Gantz I, Konda Y, Tashiro T, Shimoto Y, Miwa H, Munzart G, Watson SJ, DelValle J, and Yamada T (1993) Molecular cloning of a novel melanocortin receptor. *J Biol Chem* 268:8246–8250.

Gantz I, Shimoto Y, Konda Y, Miwa H, Dickinson CJ, and Yamada T (1994) Molecular cloning, expression and characterization of a fifth melanocortin receptor. *Biochem Biophys Res Commun* 200:1214–1220.

Gonzalez MI, Vaziri S, and Wilson CA (1996) Behavioral effects of alpha-MSH and MCH after central administration in the female rat. *Peptides* 17:171–177.

Guimaraes FS, Carobrez AP, De Aguiar JC, and Graeff FG (1991) Anxiolytic effect in the elevated plus-maze of the NMDA receptor AP7 microinjected into the dorsal periaqueductal grey. *Psychopharmacology* 103:91–94.

Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, et al. (1997) Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131–141.

Kask A, Mutulis F, Muceniece R, Pahkla R, Mutule I, Wikberg JE, Rago L, and Schioth HB (1998) Discovery of a novel superpotent and selective melanocortin-4 receptor antagonist (HS024): evaluation in vitro and in vivo. *Endocrinology* 139: 5006–5014.

Millan MJ, Dekeyne A, Papp M, La Rochelle CD, Macsweeny C, Peglion JL, and Brocco M (2001) S33005, a novel ligand at both serotonin and norepinephrine transporters. II. Behavioral profile in comparison with venlafaxine, reboxetine, citalopram and clomipramine. *J Pharmacol Exp Ther* 298:581–591.

Millan MJ, Lejeune F, Gobert A, Brocco M, Auclair A, Bosc C, Rivet JM, Lacoste JM, Cordi A, and Dekeyne A (2000) S18616, a highly potent spiroimidazoline agonist at α2-adrenoceptors: II. Influence on monoaminergic transmission, motor function and anxiety in comparison with dexmedetomidine and clonidine. *J Pharmacol Exp Ther* 295:1206–1222.

Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, and Cone RD (1994) Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol Endocrinol* 8:1298–1308.

Okuyama S, Chaki S, Kawashima N, Suzuki Y, Ogawa S, Nakazato A, Kumagai T, Okubo T, and Tomisawa K (1999) Receptor binding, behavioral and electrophysiological profiles of nonpeptide corticotropin-releasing factor subtype 1 receptor antagonists CRA1000 and CRA1001. *J Pharmacol Exp Ther* 289:926–935.

Owen MJ and Nemeroff CB (1991) Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol Rev* 43:425–473.

Panksepp J and Normansell L (1990) Effects of ACTH (1–24) and ACTH/MSH (4–10) on isolation-induced distress vocalization in domestic chicks. *Peptides* 11:915–919.

Pigott TA and Seay SM (1999) A review of the efficacy of selective serotonin reuptake inhibitors in obsessive-compulsive disorder. *J Clin Psychiatry* 60:101–106.

Porsolt RD, Anton G, Blavet N, and Jalfre M (1978) Behavioral despair in rats: a new model sensitive to antidepressant treatments. *Eur J Pharmacol* 47:379–391.

Roselli-Rehfuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, Entwistle ML, Simerly RB, and Cone RD (1993) Identification of a receptor for γ melanocortin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci USA* 90:8856–8860.

Schioth HB, Mutulis F, Muceniece R, Prusis P, and Wikberg JES (1998) Discovery of novel melanocortin$_4$ receptor selective MSH analogues. *Br J Pharmacol* 124:75–82.

Takamori K, Kawashima N, Chaki S, Nakazato A, and Kameo K (2001a) Involvement of corticotropin-releasing factor subtype 1 receptor in the acquisition phase of learned helplessness in rats. *Life Sci* 69:1241–1248.

Takamori K, Yoshida S, and Okuyama S (2001b) Repeated treatment with imipramine, fluvoxamine and tranylcypromine decreases the number of escape failures by activating dopaminergic systems in a rat learned helplessness test. *Life Sci* 69:1919–1926.

Vergoni AV, Bertolini A, Wikberg JE, and Schioth HB (1999) Selective melanocortin MC4 receptor blockage reduces immobilization stress-induced anorexia in rats. *Eur J Pharmacol* 369:11–15.

Von Frijtag JC, Croiset G, Gispen WH, Adan RA, and Wiegant VM (1998) The role of central melanocortin receptors in the activation of the hypothalamus-pituitary-adrenal-axis and the induction of excessive grooming. *Br J Pharmacol* 123:1503–1508.

Vrinten DH, Adan RAH, Groen GJ, and Gispen WH (2001) Chronic blockade of melanocortin receptors alleviates allodynia in rats with neuropathic pain. *Anesth Analg* 93:1572–1577.

Vrinten DH, Gispen WH, Groen GJ, and Adan RAH (2000) Antagonism of the melanocortin system reduces cold and mechanical allodynia in mononeuropathic rats. *J Neurosci* 20:8131–8137.

Address correspondence to: Dr. Shigeyuki Chaki, Psychiatric Diseases and Pain Research,, Medicinal Pharmacology Laboratory, Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-cho, Saitama, Saitama 330-8530, Japan. E-mail: s.chaki@po.rd.taisho.co.jp